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ADP, CDP, GDP, IDP, UDP	5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine and uridine
AMP etc.	Adenosine 5′-phosphate etc.
ATP etc.	Adenosine 5′-triphosphate etc.
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
DPN	Diphosphopyridine nucleotide
EDTA	Ethylenediaminetetra-acetate
FMN	Flavin mononucleotide
FAD	Flavin–adenine dinucleotide
GSH, GSSG	Glutathione, reduced and oxidized
NAD	Nicotinamide–adenine dinucleotide
NADP	Nicotinamide–adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
RNA	Ribonucleic acid
TPN	Triphosphopyridine nucleotide
tris	2-Amino-2-hydroxymethylpropane-1:3-diol

Abbreviations for amino acids and carbohydrates. Those marked * and † in *Suggestions to Authors* are to be used only for representation of polymers or sequences and in Tables and Figures.

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Acid-Soluble Phosphate Metabolism in Nuclei from Rat Thymus Gland

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The discovery of nuclear phosphorylation and of the ability of isolated nuclei to incorporate protein and nucleic acid precursors (Allfrey, Mirsky & Osawa, 1955, 1957*a*) focused attention on the part that might be played by the nucleus in the metabolism of the whole cell. Anderson (1953) from his work on the permeability of isolated calf-thymus nuclei to proteins concluded that quite large molecules could enter the nucleus. On the other hand, thymus nuclei isolated in an aqueous medium retained nucleotides (Allfrey *et al.* 1955) so that some process might be envisaged by which precursor molecules are held in the nucleus before the formation there of new molecules. We therefore attempted to define more precisely the relationship between acid-soluble phosphate metabolism in the nucleus and that in the whole cell and this led to some observations on uptake of inorganic phosphate and arginine by nuclei from rat-thymus glands which may be relevant in other types of animal cells.

MATERIALS AND METHODS

Animals weighing between 80 and 130 g. were taken from the laboratory stock of Wistar rats. Most of the experiments were made with thymus glands from females, but no obvious difference was found between preparations from either sex. Unless otherwise stated, the animals were injected intramuscularly with 25–50 μc of ^{32}P/100 g. body wt. 5–60 min. before death.

Isolation of nuclei. The sucrose medium A of Creasey & Stocken (1959) and their ionic medium C were used, except that 5 mM-tris replaced the 5 mM-triethanolamine hydrochloride in medium A. Medium C: 45 mM-glucose, 85 mM-KCl, 8·5 mM-NaCl, 2·5 mM-$CaCl_2$, 2·5 mM-$MgCl_2$, 5 mM-triethanolamine hydrochloride adjusted to pH 7·2 with KOH. Neither medium contained acid-soluble phosphate. The homogenization was performed in a tube of 0·5 in. diameter with the homogenizer described by Philpot & Stanier (1956) and all operations were carried out at $\pm 2°$. The nuclei were washed twice before final resuspension. The method of removal of the thymus and the time that this took were critical, as was the concentration of the thymus during the first homogenization. These details are described in the text.

Cell sap. The supernatant from the first sedimentation of the nuclei was centrifuged for a further 60 min. in the Spinco centrifuge model L (rotor 30, 30 000 rev./min.; rotor 40, 40 000 rev./min.), and the supernatant from this was used for the determination of the specific activity of the inorganic phosphate of the cell sap.

Isolated thymocytes. These were obtained as described earlier (Ord & Stocken, 1958*a*). They were suspended in ionic medium C in which the Na^+ and K^+ ion concentrations were reversed.

Acid-soluble phosphate analysis. In some experiments the reactions were stopped by the addition of $HClO_4$ to give a solution N with respect to $HClO_4$. The precipitate was separated by centrifuging and the supernatant fluid filtered before analysis. Usually the nuclear suspension was centrifuged before acidification, and the supernatant separated, made N with respect to $HClO_4$ and then filtered. The phosphate compounds present in this fraction are referred to as the soluble or supernatant fraction of the nuclei. The residue from the nuclear suspension was redispersed in medium and acidified to give the acid-extractable or bound-phosphate fraction.

Inorganic phosphate was estimated by the method of Berenblum & Chain (1938). Acid-labile phosphate was that liberated by heating for 15 min. in N-HCl at 100°. When the sucrose medium was used a yellow colour appeared on hydrolysis, but this did not interfere with the measurement of the inorganic phosphate.

In all experiments the samples were brought to a final volume of 10 ml. with *iso*butanol and filtered before estimation of the inorganic [^{31}P]phosphate and ^{32}P. When inorganic phosphate of the acid-extractable nuclear fraction was assayed a precipitate was produced in the presence of the phosphomolybdic acid. This was retained in the *iso*butanol extract which was assayed for ^{32}P before and after filtration. The ^{32}P content of the precipitate could then be obtained by difference. Direct measurement of its ^{31}P content was not always performed since experiments showed that it contributed only a small amount to the ^{31}P in the acid-extractable, acid-labile fraction.

Arginine. This was estimated by the method of Rosenberg, Ennor & Morrison (1956).

Hexokinase. The enzyme was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. (yeast enzyme, crude type II), and used to determine the specific activity of the γ-phosphate group of ATP.

Separation of ribonucleotides. This was achieved chromatographically on Dowex 1 (formate form; X10) columns (Hurlbert, Schmitz, Brumm & Potter, 1954).

Estimation of deoxyribonucleic acid. The method of Burton (1956) was used. A sample from the $HClO_4$ extract was taken for colorimetric assay and the remainder was made up to 10 ml. so that the ^{32}P activity in the DNA could be obtained.

Measurement of radioactivity. ^{32}P activity was determined in a Geiger–Müller M6 liquid counter. ^{14}C activity was measured in a Nuclear-Chicago Corp. counter. Sufficient counts were recorded to give an error of less than 3%.

RESULTS

Isolation of nuclei. Considerable care is required to obtain reproducible conditions for studying nuclear behaviour (Allfrey, Mirsky & Osawa, 1957*b*). It was therefore necessary to define as closely as possible the conditions under which the nuclei used in this work were prepared. We found that factors leading to a good yield of nuclei, containing a reproducible amount of acid-soluble phosphate, appeared to be similar for both the sucrose and the ionic media. With sucrose it was essential to increase the $CaCl_2$ concentration above 1·8 mM used by Hogeboom, Schneider & Striebich (1952) for liver nuclei; 3·3 mM-$CaCl_2$ (Allfrey *et al.* 1955) gave good yields of nuclei which were very little contaminated by whole cells. The pH of both media at room temperature was between 7·15 and 7·25 (glass electrode); no pH outside this range was used.

If the thymus was not removed rapidly from the animal at death, a considerable increase in nuclear inorganic phosphate occurred mainly at the expense of acid-labile phosphate (Table 1). Even the 30 sec. necessary for exsanguination decreased the nuclear acid-labile phosphate. The properties of the nuclei were found to be very dependent on the relative proportions of inorganic and acid-labile phosphate which they contained when first isolated.

Attempts were made to increase the yield of nuclei by taking a greater number of thymus glands in the initial homogenization. This proved to be unsatisfactory, especially with the sucrose medium, when the homogenization was always less easy than with the ionic medium. Examination of the sucrose nuclei isolated from a concentrated homogenate showed a considerable leakage of the DNA and a reduced yield owing to poor sedimentation from the more concentrated homogenates.

Unless otherwise stated the final conditions chosen for the preparation of the nuclei were the rapid removal of the thymus from rats killed by cervical fracture and the homogenization of not more than six glands at a time. Usually the twice-washed nuclei from two such operations were pooled to give the final nuclear suspension. Eight consecutive experiments in ionic medium gave: inorganic phosphate, $1·82 \pm 0·46$ μg./mg. of DNA P; acid-labile phosphate, $9·86 \pm 2·32$ μg./mg. of DNA P.

Properties of isolated nuclei. The retention of nucleotides by thymus nuclei has been discussed by Allfrey *et al.* (1955). In our experiments the final nuclear suspension, when recentrifuged, gave a supernatant containing inorganic but not acid-labile phosphate. This inorganic phosphate was about 50% of the total inorganic phosphate of the suspension (Table 2). Treatment of the nuclei with acid was required to extract the remaining inorganic phosphate and the acid-labile phosphates. In nuclei from thymus glands that had aged within the rats after death the increased inorganic phosphate was distributed between both the soluble and acid-extractable fractions. From an approximately 1:1 ratio in nuclei from thymus glands removed immediately after death, the distribution shifted towards a bound:soluble ratio of 1·5:1 (Table 2).

Chromatographic separation of the nucleotides in the acid-extracted fraction of the nuclei, from thymus glands which had been rapidly removed, confirmed the earlier findings of Allfrey *et al.* (1955) that adenine nucleotides predominated. In our

Table 1. *Effect on the nuclear acid-soluble phosphates of the time after death when the thymus was removed*

Time (min.) after death (and no. of experiments)	Acid-soluble phosphates (μg. of P/mg. of DNA P)			
	Ionic medium		Sucrose medium	
	Inorganic	Acid-labile	Inorganic	Acid-labile
< 0·3 (5)	1·7	10·9	1·8	10·1
0·5 (3)	4·3	8·9	—	—
5 (2)	5·6	7·7	3·6	6·9
10 (3)	7·1	4·8	—	—
20 (1)	13·4	4·1	7·5	3·8

Table 2. *Distribution of acid-soluble phosphates in isolated rat-thymus nuclei*

Time (min.) of thymus removal after death (and no. of experiments)	Acid-soluble phosphate					
	Total (μg. of P/mg. of DNA P)		% present in			
			Soluble fraction		Bound fraction	
	Inorganic	Acid-labile	Inorganic	Acid-labile	Inorganic	Acid-labile
< 0·5 (3)	2·8	7·8	54	0	46	100
10–15 (4)	6·0	5·8	40	8	60	92

Table 3. *Specific activities of acid-soluble phosphates in nuclei from rat thymus after injection of 50 μc of ^{32}P/100 g. body weight*

Time (min.) after injection (and no. of experiments)	Mean specific activities (counts/min./μg. of P)			
	DNA	Soluble inorganic	Bound inorganic	Acid-labile
5 (1)	0·4	97	158	—
10 (2)	0·8	187	341	133
20 (5)	1·6	310	431	259
30 (2)	3·4	280	425	260
60 (4)	6·2	125	276	222
120 (1)	—	—	218	213
240 (1)	—	—	190	158

Table 4. *Specific activities of inorganic phosphate and adenosine triphosphate from whole rat thymus and from cell fractions of the gland*

The rats received 50 μc of ^{32}P/100 g. body weight except †, where approx. 40 μc were used. The nuclear inorganic phosphate was the 'bound' fraction.

Time after injection (min.)		Specific activity of phosphate (counts/min./μg. of P) in Cell fraction			Whole thymus		
		Inorganic	Acid-labile	γ-P of ATP	Inorganic	Acid-labile	γ-P of ATP
5	Cell sap*	146	99	105	210	75	104
5	Cell sap	118	—	—	224	91	96
6	Nuclei	175	39	73	232	114	171
10		—	—	—	258	175	208
10†	Nuclei	185	77	79	212	116	102
10†	Nuclei	169	70	80	230	107	102
20	Cell sap	288	207	—	335	262	299

* In this experiment a Ca^{2+}-free medium was used (see text).

extracts ATP and AMP were the main components, very little ADP being present. A small amount of guanosine triphosphate and uridine triphosphate was found, but it was evident that about 70 % of the acid-labile phosphate was ATP. This was confirmed in the course of hexokinase assays of the specific activity of the γ-phosphate group of ATP (see below). In these rat nuclei, in contrast with those from calves but in agreement with the findings from whole rat thymus (Ord & Stocken, 1958*b*), cytidylic acid was only slightly lower in concentration than AMP. In extracts from rats given ^{32}P 30 min. before death, the activity of ATP was greater than in ADP, and AMP had insignificant counts.

Specific activity of inorganic phosphate in different cell fractions. It was observed in these experiments, in which the rats had received ^{32}P at various times before death, that the specific activity of the acid-extractable inorganic phosphate of the nucleus was higher than either the soluble inorganic phosphate or the acid-labile phosphate of the nucleus (Table 3). Determination of the specific activities of inorganic and acid-labile phosphates in the cell sap apparently indicated that the specific activity of bound inorganic phosphate in the nucleus was higher than the inorganic phosphate in the cell sap at times between 5 and 60 min. after injection. It was, however, noticed that the amount of acid-labile phosphate in the cell sap was appreciably lower than that found earlier (Ord & Stocken, 1958*b*) when whole rat thymus was analysed. This suggested that, in the course of fractionation at 0°, breakdown of less highly labelled compounds had taken place, so diluting the specific activity of the inorganic phosphate. When the activity was determined on extracts from the whole gland, frozen in liquid air immediately after removal, much higher specific activities for the inorganic phosphate were found (Table 4).

Assays of the γ-phosphate group of ATP indicated that ATP in the nucleus was not identical with that from the whole extract. The activity of the γ-phosphate group in the whole extract was initially lower than that of the inorganic phosphate; dilution from the nucleus could have occurred although the amount of ATP in the nucleus is less than in the cytoplasm. It did not prove possible to isolate mitochondria containing ATP from the thymus although a Ca^{2+}-free medium was used (Bellamy & Bartley, 1960; 0·15 M-KCl–1 mM-tris–1 mM-EDTA), which had previously given good results for oxidative phosphorylation with thymus mitochondria. In cell sap obtained after sedimentation of the mitochondria in the Ca^{2+}-free medium the specific activity of the γ-phosphate group was identical with that from the whole thymus.

To find out why the nuclei retained their highly active inorganic phosphate during fractionation and to explore further the interactions between nuclear and cytoplasmic ATP and P, uptake of ^{32}P was studied *in vitro* with thymus nuclei and thymocytes.

Nature of the inorganic phosphate binding by the nucleus

'Soluble' fraction of the nucleus. The reality of the division of this phosphate into at least two components was based mainly on the physical separation of two inorganic phosphate fractions of different specific activities (Table 3). Since the soluble, low-specific-activity inorganic phosphate was obtained after acidification of the supernatant from the centrifuged nuclear suspension, and since this acidification produced a fairly copious white precipitate, it was possible that the inorganic phosphate had been split off from some substance(s) in the supernatant. This was tested by dialysing the supernatant from nuclei isolated in ionic medium against 2 or 3 vol. of medium at 0° (Table 5).

It was clear from determination of the total counts in the soluble fraction at the start and from the counts attributable to inorganic phosphate, that not all the ^{32}P activity was due to material giving inorganic phosphate after treatment with cold N-$HClO_4$. ^{32}P could be extracted from the white precipitate by 0·5N-$HClO_4$ at 70° and the extract showed traces of deoxyribose. This suggested that some DNA nucleoprotein was present in the soluble fraction, either as a result of leakage from the nuclei or because of poor sedimentation.

It was also evident from the specific activity of the dialysable inorganic phosphate that this inorganic phosphate was identical with the inorganic phosphate bound to the nucleus. Since the specific activity of the material within the sac fell as a result of dialysis, it seemed that the inorganic phosphate of the 'soluble' fraction was made up of two components: a dialysable fraction and a fraction of comparatively low specific activity which gave inorganic phosphate after treatment with acid. The nature of this non-dialysable component has not been studied further, although it was noted that incorporation of ^{32}P into the soluble fraction was inhibited by iodoacetate (Table 10).

The dialysable fraction apparently represented inorganic phosphate of high specific activity, which was either dissociated from its nuclear binding in the last stages of the isolation procedure or was normally trapped within the nucleus.

'Bound' inorganic phosphate of the nucleus. This fraction had been recognized because of its high specific activity when nuclei were isolated from animals given ^{32}P 5–60 min. previously. It was of interest to determine whether the nuclear suspension, after the 'soluble fraction' had been separated by centrifuging, would take up the isotope when shaken gently in ionic medium containing ^{32}P. Preliminary experiments established that resuspending the nuclei three times in unlabelled medium reduced the ^{32}P of the supernatant to a low level. Further washing appeared to cause breakdown of the nuclei, as shown by the appearance in the supernatant of acid-labile phosphate. It is clear that (Table 6) the specific activity of the bound inorganic phosphate after incubation of the nuclei with ^{32}P *in vitro* was much greater than that of the other two fractions examined and that there was no difference between the uptake at 0° and 37°, although it is possible that the nuclei were only able to tolerate rather short incubations at 37°. Additional experiments at 0° indicated that the uptake of ^{32}P into the bound inorganic phosphate was linear from 0 to 30 min. If nuclei from animals that had been given ^{32}P were incubated similarly in ionic medium containing 1 μmole of inorganic [^{31}P]phosphate/ml. (Table 7) and the soluble and bound fractions separated, there was a fall in the specific activity of the bound inorganic phosphate fraction. The change in specific activity was not as marked as with the uptake experiments but the specific

Table 5. *Effect of dialysis against ionic medium on the 'soluble' phosphate component of nuclei from rat thymus*

Specific activities are given as counts/min./μg. of P. Expt. 1: the mean of 2 experiments with rats given 100 μc of ^{32}P/100 g. body weight 1 hr. before death. Expt. 2: rats given 50 μc of ^{32}P/100 g. body weight 20 min. before death.

	Expt. no. 1	Expt. no. 2
At start		
Total counts in sac	3754	718
Counts due to inorganic phosphate	2020	423
Total inorganic [^{31}P]phosphate (μg.)	4·03	1·46
Specific activity of inorganic phosphate	502	289
Specific activity of bound inorganic phosphate	653	373
Ratio volume of sac: volume of diffusate	1:2·5	1:3
After dialysis for 2 hr. at 0°		
Counts in sac	2426	406
Specific activity of dialysable inorganic phosphate	663	359
Amount of dialysable inorganic phosphate (μg.)	2·01	0·78
Specific activity of non-dialysable material giving inorganic phosphate on treatment with N-$HClO_4$	424	214

Table 6. *Specific activities of acid-soluble phosphates from rat-thymus nuclei labelled* in vitro *in ionic medium*

Specific activities are given as counts/min./μg. of P.

Time of incubation (min.)	Incubation at 0°			Incubation at 37°		
	Last wash	Bound inorganic phosphate	Acid-labile phosphate	Last wash	Bound inorganic phosphate	Acid-labile phosphate
30	760	2 055	66	—	—	—
30	240	1 580	120	264	1 357	91
10*	5 510	28 420	—	4530	29 950	—
30*	19 710	55 000	2740	3158	23 250	1131

* 1 μc of ^{32}P present/ml.

Table 7. *Specific activities of inorganic phosphate from rat-thymus nuclei labelled* in vivo *and incubated* in vitro *in ionic medium at 0° with inorganic* [^{31}P]*phosphate* (1 μ*mole/ml.*)

Time (min.)	Specific activities of phosphate (counts/min./μg. of P)	
	Soluble inorganic	Bound inorganic
0	6·44	191
30	8·44	161
0	2·57	72·1
10	2·96	71·5

Table 8. *Effect of extraction with* M*-sodium chloride on the 'bound' inorganic phosphate fraction from rat thymus gland*

Specific activities are given as counts/min./μg. of P. In Expts. 1 and 2 the M-NaCl extracts were dialysed for 2 hr. at 0° against 2 vol. of M-NaCl; in Expt. 3 11 vol. of M-NaCl was used.

	Expt. no.		
	1	2	3
Specific activity of bound inorganic phosphate	650	655	—
Specific activity of inorganic phosphate after acidification of M-NaCl extract	671	500	—
Specific activity in DNA of M-NaCl extract	—	9·6	—
Total inorganic phosphate in M-NaCl extract (μg.)	2·54	1·6	4·32
Inorganic phosphate dialysable (%)	41	44	66·6
Specific activity of inorganic phosphate in diffusate	—	532	—

activity of the inorganic phosphate of the nucleus was about 30 times that of the supernatant as compared with an extremely high activity in the supernatant during incorporation *in vitro*.

The fact that the bound inorganic phosphate fraction was removed after treatment with acid suggested that an ionic linkage might be involved and this is supported by the high rate of exchange of the phosphate at 0°. The possibility that the inorganic phosphate might be associated with a lipid component was investigated by using the $CHCl_3$–methanol procedure described by Getz & Bartley (1961). Shaking the lipid solution with N-$HClO_4$ did not liberate appreciable inorganic [^{31}P]- or [^{32}P]-phosphate into the aqueous phase.

It was therefore decided to investigate extraction procedures that had been applied to nuclei in other work. Allfrey *et al.* (1957*a*) have described the sensitivity of nucleotide retention by calf-thymus nuclei to extraction with 0·02 M-acetate buffer, pH 4·5–5·0. This was confirmed in our experiments but short-time treatment with acetate buffer, comparable with that with N-$HClO_4$, was less effective in dissociating the phosphate and nucleotides than acid extraction.

Extraction of nuclei, from which the soluble fraction had been removed, by M-NaCl at 0° (Allfrey *et al.* 1957*a*) led to the isolation of a clear, viscous fraction, which on treatment with N-$HClO_4$ gave inorganic phosphate of the same specific activity as in the original nuclei, in about 70 % yield (Table 8). Although there was a considerable purification on a DNA basis the extraction was by no means complete and there were indications that inorganic phosphate could be obtained from the residue after centrifuging off the viscous supernatant. The specific activity of the inorganic phosphate obtained by acidification of the M-NaCl extract was enormously greater than that of the DNA present in the same fraction. Dialysis of the non-acidified material for 2 hr. against M-NaCl showed that 40 % of the inorganic phosphate bound in the sol was dialysable. When dialysed against a larger volume of M-NaCl a greater proportion of inorganic phosphate passed through the membrane, and it would appear therefore that the complex with inorganic phosphate was dissociable by solutions of high ionic strength.

Arginine uptake by nuclei

In some experiments analyses were made for components other than phosphates in the acid extracts from the nuclei or nucleoprotein. Chro-

Table 9. *Uptake of* ^{32}P *by thymocyte suspensions* in vitro

Control and experimental incubations were for 15 min. at 37° unless otherwise stated. In all the experiments 0·3 μc of ^{32}P/ml. was present. The concentration of iodoacetate was 1 mM.

			Specific activities of phosphate (counts/min./μg. of P)				
			Nuclei			Cell sap	
	10^{-6} × Cells/ml.	Free nuclei (%)	Total inorganic	Soluble inorganic	Bound inorganic	Inorganic	Acid-labile
Control	26·9	19	575	—	—	224	103
15 min. at 0°			229	—	—	7	3
Control	51·2	6	256	—	—	183	105
Iodoacetate–O_2			242	—	—	198	211
Control	48·6	0	—	100	259	256	135
Iodoacetate–N_2			—	34	93	98	41
Control	53·7	20	—	483	601	490	297
Iodoacetate–N_2			—	143	661	69	45
Control*	23	36	—	595	772	502	253
Iodoacetate–N_2			—	500	900	222	104

* Rabbit thymocytes used.

matographic evidence indicated the presence of arginine, so that the possibility of nuclear binding of arginine was also investigated. Allfrey & Mirsky (1959) have provided evidence that amino acid incorporation into protein in calf-thymus nuclei is likely to be through an activated amino acid–RNA complex similar to that in microsomal protein synthesis. Hoagland, Zamecnik & Stephenson (1957) established that amino acid–soluble RNA complexes were not broken down by cold 0·4 N-$HClO_4$. It therefore seemed probable that the arginine found in the extracts was not derived from breakdown of an arginine–RNA complex. Estimation of the arginine:inorganic+acid-labile phosphate ratios in the 'bound' fraction from isolated nuclei showed the presence of about 0·5 mole of arginine/mole of total inorganic+acid-labile phosphate. Incubation of the nuclei in ionic medium at 0° with [^{14}C]arginine showed a linear uptake. [^{14}C]Arginine (50 000 counts/min.) was added to nuclei from 12 thymus glands suspended in 14 ml. of ionic medium. At 0, 5 and 15 min. incubation at 0° the specific activities of the bound arginine were 1100, 1745 and 2880 counts/min./μmole. The rate of uptake appeared to be similar to that for inorganic phosphate.

Uptake of ^{32}P *by thymocyte suspensions*

Suspensions of whole cells are comparatively easy to obtain from rat thymus (Ord & Stocken, 1958*a*). The thymocytes can be distinguished from naked nuclei by examination with phase-contrast microscopy in albumin solution (Barer, Joseph & Esnouf, 1956). After incubation with ^{32}P the suspensions were immediately diluted with 20 vol. of medium at 0° and washed three times. The cells were suspended in a small volume of medium C and homogenized with a plunger of 0·002 in. clearance. The nuclei were then washed in the usual way. The first supernatant obtained after sedimentation of the nuclei was centrifuged at 10 000*g* and the specific activities of inorganic and acid-labile phosphate were determined.

Uptake of ^{32}P into the cell sap was negligible at 0° although the nuclear inorganic phosphate became labelled. In all the experiments the specific activity of the nuclear inorganic phosphate exceeded that of the cell sap by an amount that seemed to be proportional to the number of free nuclei present in the original suspension (Table 9). This was confirmed by incubating in the presence of 1 mM-iodoacetate. Preliminary experiments showed that 1 mM-iodoacetate had no effect on ^{32}P uptake into the bound inorganic phosphate of isolated nuclei; with thymocyte suspensions iodoacetate under anaerobic conditions reduced the uptake into the cell sap by about 70% but where free nuclei were present their uptake was unaffected or slightly increased. In the experiment with iodoacetate under aerobic conditions ^{32}P uptake into the cell sap was not decreased.

DISCUSSION

The interpretation of the results presented here rests on the purity of the nuclear fraction and on the accuracy of the phosphate determinations. Preparations of nuclei were examined by phase-contrast microscopy and after staining. Very few whole cells were found and there was very little contamination from cytoplasmic debris or red cells. The majority of the nuclei were small and round but about 10% were larger and less regular in shape. Some of the nuclei, especially larger ones, had tags of cytoplasm attached. Classical radioautography does not enable the presence of water-soluble compounds in nuclei to be detected; a technique has,

however, been devised permitting the retention of these compounds (Fitzgerald, Ord & Stocken, 1961). By this means it has been possible to show, in nuclei from thymus glands of rats given the isotope *in vivo*, ^{32}P that was not present in nuclei that had been washed with N-perchloric acid or fixed by the usual methods. A detailed examination of the nuclei has not so far been made so that it is not possible to say whether the uptake is a feature of all types of nuclei at all stages of their development.

Ionic medium was used for the isolation of nuclei in most of the experiments with thymus, and no difference in properties has been found between nuclei prepared in this medium and those in sucrose. We have found that nuclei in ionic medium were easier to prepare and handle and appeared to tolerate the final resuspension to a small volume better than in sucrose.

The amount of inorganic phosphate in the nuclear suspensions was very low so that quantities of 0·5 μg. were sometimes estimated. It was difficult to increase the amount assayed because of the limit to the number of thymus glands that could be used in the preparation of the nuclei. In nuclei from thymus glands that had been removed immediately after death, where the total inorganic phosphate was particularly low, the amount of inorganic phosphate in the 'soluble' and 'bound' fractions was approximately the same but the difference in ^{32}P activity was very marked. The accuracy of the ^{32}P determinations was $\pm 3\%$.

Much of the pioneering work on uptake of ^{32}P by tissues was carried out by Hevesy (1948) and his colleagues and he and Marshak (1941) were the first to study incorporation into the nuclear fraction of cells. The isolation procedure entailed a low pH and little activity was detected in the residual acid-soluble fraction. Recent work on yeast (Jennings, Hooper & Rothstein, 1958) and erythrocytes (Glynn, 1957) has shown that the entry of inorganic phosphate into these cells is markedly dependent on glycolysis and that initially glycolytic intermediates and ATP have higher specific activities than intracellular inorganic phosphate. Sacks & Altshuler (1942) studied the uptake of phosphate by cardiac and striated muscle and concluded that it was first converted into organophosphate. In Ehrlich ascites-tumour cells Wu & Racker (1959) found that, whereas addition of glucose stimulated the entry of inorganic phosphate, in the absence of glucose the entry appeared to be by an exchange diffusion that was unaffected by iodoacetate. Our experiments on the entry of inorganic phosphate into the cytoplasm of thymocytes lead to a similar interpretation, and it seems that in these cells uptake of inorganic phosphate may be linked to glucose utilization but does not occur exclusively through glycolytic intermediates.

The reversible uptake of inorganic phosphate by nuclei at 0°, the absence of a temperature effect on the reaction, its insensitivity to 1 mM-iodoacetate and the removal of the inorganic phosphate by treatment with cold, dilute acid suggest that the inorganic phosphate is bound to the nucleus in an ionic linkage. The site of the binding remains obscure. Association with histones seems probable and is consistent with the extraction of the bound material by M-sodium chloride. Arginine binding might be expected since Mirsky & Ris (1951) and Davison & Butler (1956) have reported the presence of free negative charges on DNA–nucleoprotein. Kuzin & Shabadash (1959) have proposed that excessive negative charges are especially evident in nuclei from lymphoid tissues. It is not yet known whether other basic amino acids are similarly bound. Na^+ ion binding in calf-thymus nuclei is indicated from the work of Itoh & Schwartz (1957), who found a reversal of the Na:K ratio from that in the cytoplasm. Preliminary experiments have shown that ^{32}P uptake by isolated rat-thymus nuclei was unaffected by the addition of arginine to the medium, suggesting either that the inorganic phosphate and arginine are taken up independently of one another or that the system is already saturated with arginine.

The relationship of the nucleoprotein described here to chromosomes or the nucleolus is unknown but the results suggest that the charged macromolecules have associated with them in the intact nucleus an ordered and reproducible array of charged, low-molecular-weight compounds. It may not be coincidence that nucleotide binding by rat liver is very low or absent (Rees & Rowland, 1961) and that so far nuclear phosphorylation, which presumes the presence of bound nucleotide acceptors, has been described only in nuclei from tissues containing dividing cells (Creasey & Stocken, 1959). It seems possible that the binding of low-molecular-weight compounds in the nucleus may be relevant to the incorporation of those same molecules into the macromolecular structure of the nucleus. However, Allfrey *et al.* (1955) found no significant inhibition of amino acid incorporation into the proteins of isolated nuclei when free phosphate groups in the DNA were blocked by various reagents.

SUMMARY

1. Some of the conditions have been defined for the isolation of nuclei from rat thymus gland containing reproducible amounts of acid-soluble phosphates. The nuclei contained 1–2 μg. of inorganic phosphate and about 10 μg. of acid-labile phosphate/mg. of deoxyribonucleic acid phosphorus.

2. ^{32}P was taken up readily by nuclei *in vivo* and *in vitro*. The process occurred rapidly at 0°, was not

accelerated by increasing the temperature to 37° and was not inhibited by 1 mM-iodoacetate.

3. The binding of inorganic phosphate by thymus nuclei was investigated. Linkage to nucleoprotein is probable but the exact nature of the complex is not yet certain.

4. Entry of inorganic phosphate to the nucleus did not require prior uptake into adenosine triphosphate. Entry into the cytoplasm was possibly linked with glucose utilization; its dependence on high-energy phosphate compounds is discussed.

5. Arginine also could be extracted from the nuclei with 0·5–1·0N-perchloric acid at 0°. The molar ratio of total inorganic and labile phosphates: arginine was approximately 2:1.

6. The arginine in the nuclei exchanged rapidly with [^{14}C]arginine at 0°.

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Phospholipid Metabolism in Nervous Tissue

4. INCORPORATION OF ^{32}P INTO THE LIPIDS OF SUBCELLULAR FRACTIONS OF THE BRAIN*

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In earlier investigations (Davison & Dobbing, 1960*a*, *b*; Davison, Morgan, Wajda & Payling Wright, 1959) it was shown, by introduction of radioactive precursors into developing animals, that part of the brain lipids remained metabolically stable during growth and subsequently in the adult animal. It was suggested that this metabolic stability was associated with the myelin sheath and possibly other anatomical structures in the brain. This concept was supported by the demonstration

* Part 3: Davison & Dobbing (1960*b*).

that stable radioactive lipid was found more in white matter than in grey (Davison & Dobbing, 1960*b*) and that radioactivity was retained only by lipids known to be present in myelin. This paper describes attempts to extend these observations by determining radioactivity incorporated into the lipids of subcellular fractions and myelin-enriched fractions from the brain of rats at intervals after injection of suitable precursors.

METHODS

In the first experiment five 16-day-old white rats from one litter were given 0·25 ml. of 0·9% sodium chloride solution containing 10 μc of ^{32}P as phosphate by intraperitoneal injection. Five rats from the same litter were injected with 0·2 ml. of 0·9% sodium chloride solution containing 8 μc of ^{35}S as sulphate. The rats were killed at various intervals after injection and the brain of each was removed, washed free of blood, dried with filter paper and weighed. The brain was then suspended (10%, w/v) in ice-cold 0·25 M-sucrose by homogenization in a MSE homogenizer for 5 min. at a predetermined speed. Two samples (0·5 ml.) of the suspension were extracted by refluxing with 10 ml. of ethanol–acetone (1:1, v/v). The mixture was filtered and the residue was re-extracted by the same procedure, and the combined filtrates were evaporated to dryness. The dry residue was extracted with 10 ml. of chloroform–methanol (2:1, v/v) and filtered. The extract was washed with 0·2 vol. of water (Folch, Lees & Sloane-Stanley, 1957) and repeatedly with synthetic upper phase (Folch *et al.* 1957) until free from radioactivity. Radioactivity in the lower phase was determined either for ^{32}P in the β-counter for liquids or for ^{35}S by scintillation counting (Davison & Dobbing, 1960*a*). Phosphorus was determined as described previously (Davison & Dobbing, 1959).

Differential centrifuging. The remaining brain suspension was fractionated by differential centrifuging as described by Brody & Bain (1952), except that only one mitochondrial fraction was prepared (i.e. R_2+R_3). Lipid was extracted from particulate fractions as described above. The supernatant was mixed with 15 vol. of ethanol–acetone (1:1, v/v) and, after refluxing for 10 min., the mixture was filtered and the filtrate taken to dryness. The dried residue was then extracted with chloroform–methanol (2:1, v/v) and washed as described above.

Fractionation in sucrose density gradients. In the second experiment four young adult rats (body weight 219–241 g.) and one older animal (329 g.) were given intraperitoneally 50 μc of ^{32}P as phosphate in 0·5 ml. of 0·9% sodium chloride solution. Two neonatal rats were injected with 0·25 ml. of 0·9% sodium chloride solution containing 10 μc of ^{32}P as phosphate. Animals were killed at stated times after injection. A myelin-rich fraction of brain was then prepared by a modification of the techniques of Korey (1957) and Whittaker (1959). Brains were suspended in 0·25 M-sucrose as described before. A portion (10 ml.) of the suspension was layered over a discontinuous density gradient consisting of 10 ml. of 0·5 M- and 10 ml. of 1·75 M-sucrose. The tubes were centrifuged at 15 000*g* at 10° for 30 min. Three layers separated: a topmost translucent layer, a middle fluffy layer and a red layer at the interface of the 0·5 M- and 1·75 M-sucrose layer. The middle fluffy layer (in approx. 5 ml. of 0·5 M-sucrose) was removed, diluted with 10 ml. of water and centrifuged for 20 min. at 12 000*g*. The supernatant from the centrifuge tube was decanted and the remaining pellet suspended in 10 ml. of 0·8 M-sucrose and centrifuged again at 25 000*g* for 2 hr. at 10°. The white material floating at the top of the centrifuge tube was separated from the supernatant and precipitate (particulate fraction) and diluted to 60 ml. with water. A pellet was then collected on centrifuging at 12 000*g* for 10 min. at 10°. Radioactivity in the original suspension and final pellet was determined as described above.

Extraction with salt solution. In a third experiment two adult rats (390 g.) were injected intraperitoneally with 0·2 ml. of 0·9% sodium chloride solution containing 60 μc of [^{32}P]phosphate. The animals were killed after 2 days and the brains removed. Each brain was suspended in 20% (w/v) sodium chloride solution at 0°, 10 ml./g. of fresh tissue (LeBaron & Folch, 1959). The suspension was then centrifuged at 30 000*g* for 40 min. at 4°. The supernatant was removed and radioactivity and lipid phosphorus were determined on extracts as described above. The brain precipitate was then separated exactly as described above in the second experiment. Lipid sulphate was estimated by the method of Long & Staples (1961).

Table 1. *Intracellular distribution of radioactive lipid in the brain of rats previously injected with* [^{32}P]*phosphate or* [^{35}S]*sulphate*

Rats (15 days old) were injected either with 0·2 ml. of salt solution containing 8 μc of [^{35}S]sulphate or with 0·25 ml. of salt solution containing 10 μc of [^{32}P]phosphate. The rats were killed at intervals after injection; the brain was removed and treated as described in the text. Figures given represent results for one rat and radioactivities are corrected for the equivalent of a dose of 1 μc of ^{32}P/g. body wt.

	^{32}P (counts/min.)		^{35}S ($\mu\mu$c)		
Time after injection	4 hr.	13 days	4 hr.	2 days	26 days
Fraction					
Whole-brain lipid extract (total radioactivity)	260	11 900	5 010	34 700	42 500
	Percentage of total brain-lipid radioactivity				
Debris and nuclei	18	33·2	6·4	11·5	12·5
Mitochondria	26·8	47·5	65·4	50	80·7
Microsomes	37·6	13·4	0·6	15·2	7·1
Supernatant	17·6	13	27	8·7	4·4

Histology. Different brain fractions were smeared on to glass slides and fixed in formalin vapour. Haematoxylin and eosin and Sudan black were used for staining. Samples for electron microscopy were fixed by the method of Chou & Meek (1958).

RESULTS

The results shown in Table 1 indicate distribution of radioactive lipid in all the cell fractions of the brains of neonatal rats 4 hr. after injection of [^{32}P]phosphate; 13 days after injection nearly half the total radioactivity was present in lipids of the mitochondrial fraction. This concentration of radioactivity in the lipids of the mitochondrial fraction was more marked when [^{35}S]sulphate was used as a precursor even 4 hr. after injection.

Histological examination showed that each brain fraction was of mixed composition and contained sudanophilic material as well as subcellular particles and debris (see also Aldridge, 1957; Petersen & Schou, 1955). Most of the lipid-staining material, probably myelin, was, however, associated with the mitochondrial fraction. The large amount of labelled sulpholipid found in the mitochondrial fraction also suggests the presence of a high proportion of myelin, for there is evidence to suggest that sulphatide is one of the most characteristic lipids of the myelin sheath (Davison, 1961). It was therefore necessary to separate the brain suspension by an alternative method in order to investigate the relationship between metabolic stability and the myelin sheath.

The method of preparation of a myelin-rich brain fraction makes it unlikely that it would contain any particulate matter; furthermore, microscopic examination showed that cells and nuclei were absent whereas characteristic staining for myelin could be demonstrated. The absence of cells and nuclei and the concentration of myelin was confirmed by electron microscopy. At low power ($\times$ 7800) in the electron microscope typical myelin forms were seen and no other particulate matter was detected; at higher magnification ($\times$ 76 250) lamellar complexes of similar periodicity to that of the intact myelin sheath were observed. (These observations were kindly undertaken by Dr C. P. Wendell-Smith.) It therefore seems that myelin-sheath fragments are concentrated in this fraction, although the extent of any contamination by lipid from other sources is not known. In the second series of experiments [^{32}P]phosphate was injected into neonatal and adult rats. The animals were killed and each brain was suspended in sucrose and separated into a myelin-enriched and a myelin-depleted fraction. Lipid was extracted from each fraction and radioactivity and lipid phosphorus were determined. Comparison of the specific activities of both fractions shows the extent of uptake

Table 2. *Distribution of radioactivity in lipids of myelin-rich fractions from the brain of rats injected with* [^{32}P]*phosphate*

Radioactivity and phosphorus estimations were as described earlier (Davison & Dobbing, 1959). The procedure for isolation of myelin concentrate is given in the text. Animals were given different doses of ^{32}P as phosphate by intraperitoneal injection. To facilitate comparison results are corrected to the equivalent of a dose of 1 μc/g. body wt. Where more than one animal was used mean figures are recorded. Specific activity = (counts/min.)/mg. of lipid phosphorus.

Age when injected	Time killed after injection (days)	Number of animals	Initial body wt. (g.)	Brain wt. (g.)	Myelin concentrate			Remainder of brain lipid			Ratio: specific activity of myelin concentrate / specific activity of remainder
					Phospholipid (mg. of phosphorus)	Radioactivity (counts/min.)	10^{-3} × Specific activity	Phospholipid (mg. of phosphorus)	Radioactivity (counts/min.)	10^{-3} × Specific activity	
13 days	2	2	25	1·28	0·131	500	3·83	1·869	6 550	3·52	1·09
14 days	36	1	25	1·65	0·38	2250	5·92	1·445	6 050	4·19	1·42
Adult	2	1	329	1·96	0·59	4420	7·5	1·54	17 580	11·4	0·66
Adult	2	4	230	1·87	0·315	1388	4·4	2·115	17 542	8·35	0·53

Table 3. *Distribution of radioactivity in the lipids of different brain fractions*

An adult rat (387 g.) was injected with 50 μc of [^{32}P]phosphate in 0·2 ml. of 0·9% sodium chloride solution. The animal was killed 2 days after injection and the brain suspended in 20% (w/v) sodium chloride solution and fractionated as described in the text. Radioactivity has been corrected to the equivalent of a dose of 1 μc/g. body wt. Specific activity = (counts/min.)/mg. of lipid phosphorus.

Brain fraction	Total phospholipid (mg. of phosphorus)	Total radioactivity (counts/min.)	10^{-3} × Specific activity	Lipid sulphate / Lipid phosphorus
Whole brain	3·24	24 400	7·15	—
Supernatant (20% NaCl)	0·174	3 240	18·6	—
'Myelin' fraction	0·238	1 350	5·67	0·59
Final particulate fraction	0·567	10 400	18·3	0·17

of [^{32}P]phosphate into each. The specific activities of myelin-enriched and myelin-depleted fractions were similar 2 days after injection of radioactive phosphate into a 13-day-old rat, but 36 days after injection there was relatively more radioactivity in the myelin concentrate from the brain (Table 2). Different results were obtained 2 days after injection of adult rats. In this case much less radioactivity was found in the lipid of the myelin concentrate than in the rest of the brain.

In the third experiment adult rats were again injected with [^{32}P]phosphate and after 2 days the brain was removed. The brain suspension was, however, first extracted with salt solution of high ionic strength to remove brain lipoprotein, then myelin concentrate and a particulate fraction were obtained as described above. During the course of the fractionation procedure much of the brain suspension is rejected (e.g. upper and lower layers after sucrose density separation), and the results shown in Table 3 therefore represent only part of the total brain myelin or particulate fraction. The ratio of lipid sulphate to lipid phosphorus was shown to be higher in the myelin-enriched fraction than in the brain particulate fraction, as would be expected if sulphatide is a characteristic myelin lipid (Davison, 1961). A typical result is shown in Table 3. In this example the specific activity of the myelin-enriched lipid fraction is about a third that in the particulate fraction and in the supernatant lipid.

DISCUSSION

In earlier papers it has been suggested that brain phospholipids, in common with other constituents, can be divided into metabolically stable and metabolically active compartments (Davison & Dobbing, 1960*a*, *b*). There is evidence to suggest from experiments on developing animals that this metabolic stability is associated with the myelin sheath and structural cellular constituents. This paper describes experiments designed to confirm the hypothesis by the use of alternative methods for fractionation of the brain. In the first investigations [^{32}P]phosphate was injected into developing rats and brain suspensions were separated by differential centrifuging into subcellular fractions. The results indicate that, 13 days after injection, the highest proportion of radioactivity was found in the mitochondrial fraction. Since the number of cerebral mitochondria increases threefold during the first 21 days after birth in the rat (Samson, Balfour & Jacobs, 1960) it could be that radioactivity was largely incorporated into developing mitochondria. But subsequent histological examination and similar experiments with [^{35}S]sulphate as precursor indicated that this fraction was of mixed composition and probably contained much of the myelin from the brain. Furthermore it seems likely that myelin fragments to a variable extent contaminated all subcellular fractions from the brain and that this contamination would vary both with the age of the animal and with the efficiency of the initial homogenization. For these reasons another type of separation was undertaken and a comparison was made between the radioactivity incorporated into myelin-enriched and myelin-depleted brain fractions.

If it be true that lipids of the myelin sheath are metabolically inert then it should be possible to incorporate ^{32}P or other suitable precursors into the lipids of this structure only during development and not subsequently. The preliminary experiments reported here are entirely consistent with the hypothesis that the metabolic activity of the myelin lipids from adult rat brain is less than that of other brain lipid. Thus a month after injection of neonatal rats, more radioactivity was found in the myelin-enriched brain fraction than in the rest of the brain phospholipid, whereas significantly less radioactivity was found in the myelin-enriched fraction from the brain of adult rats, previously injected with ^{32}P, compared with the radioactivity in the remainder of the brain (Table 2). An increased difference can be demonstrated if lipoprotein is first removed from the brain suspension: in adult rats three times more radioactivity is present in lipoprotein lipid and the brain particulate fraction than in the myelin-enriched phospholipid fraction. Nevertheless, it is clear from Tables 2

and 3 that a significant amount of radioactive phosphorus is incorporated into the myelin-enriched fraction obtained from the brain of adult rats. However, the method of preparation of the myelin-enriched brain fraction described above is necessarily crude and the criteria for homogeneity, both chemical and microscopic, are only qualitative. To this extent therefore these experiments have failed to give a clear-cut answer and further investigations with more refined methods will be necessary to determine whether or not myelin lipids and other brain structural constituents are completely inert in the adult animal.

SUMMARY

1. Radioactive phosphate and sulphate have been injected into neonatal rats.

2. Most of the persistent radioactivity is found in the lipids of the brain mitochondria, but this fraction also probably contains myelin.

3. In other experiments radioactive phosphorus as phosphate was injected into neonatal and adult rats and a myelin-enriched fraction isolated from the brain.

4. Relatively more radioactive lipid was found in the myelin-enriched fraction from the brains of neonatal rats than in the rest of the brain lipid.

5. In contrast less radioactive phospholipid was obtained from the myelin-enriched fraction from adult rats than from the remaining brain lipid.

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An Electrophoretic Investigation of Interactions between Bovine Plasma Albumin and Charged Dextran Derivatives

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The formation of soluble complexes through the interactions of macromolecules bearing net charges of the same sign is a phenomenon of considerable interest. Particular examples of this type of complex may be found in the antigen–antibody system (e.g. Singer & Campbell, 1955*a*, *b*, *c*; Singer, Eggman & Campbell, 1955), the polymerization of acid insulin (Oncley, Ellenbogen, Gitlin & Gurd, 1952; Doty & Myers, 1953), the polymerization of α-chymotrypsin (Kegeles & Rao, 1958), the interaction between nucleic acids and proteins (Goldwasser & Putnam, 1950) and the interactions of plasma proteins with charged polysaccharides (Walton, 1952; Noguchi, 1956; Bernfeld, Donahue & Berkowitz, 1957; Sasaki & Noguchi, 1959).

The work reported in this paper is an investigation, with free-boundary electrophoresis, of the effect of variations of pH, ionic strength and molecular size on the formation of soluble complexes between bovine plasma albumin and synthetic derivatives of dextran. Two types of dextran derivatives have been employed: a sulphate ester and an ether-linked diethylaminoethyl derivative. Interactions between the bovine plasma albumin

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and the dextran sulphate derivatives have been studied in the pH range above the isoelectric point of the albumin, and interactions between this protein and the amine dextrans have been studied below the albumin isoelectric point.

EXPERIMENTAL

Materials

The properties of the sulphate and diethylaminoethyl (DEAE) derivatives of dextran used in this study are summarized in Table 1. The native dextran, a glucose polymer, was partially hydrolysed with acid and the hydrolysate fractionated with acetone to yield a series of dextrans differing in mean molecular weight. Selected acetone fractions were then converted either into the sulphate ester (Ricketts, 1952) or into the DEAE derivatives (McKernan & Ricketts, 1959, 1960). The mean molecular weights of the parent dextran fractions were determined from intrinsic viscosity measurements, with the equation of Wales, Marshall & Weissberg (1953). From these values and the sulphur or nitrogen content of the substituted dextrans, the mean molecular weights of the derivatives were obtained (McKernan, 1958.)

At I 0·10, 20°, the sulphate esters have a titration constant of 2·0 and the DEAE derivatives a titration constant of 8·8. Both types of dextran derivative are stable under all conditions employed in this study.

Two lots (BA-0170 and BE-1370) of Armour crystalline bovine plasma albumin were used in this study. Both preparations behaved electrophoretically as homogeneous solutes except in the range pH 3·5–4·5. In this pH range two components were observed (cf. Aoki & Foster, 1956). The two preparations were examined in the Spinco analytical ultracentrifuge at pH 7·4, I 0·10, 5·0°. Lot BA-0170 contained about 5% of a fast component just visible as a shoulder on the main peak. Lot BE-1370, however, showed only a trace of a similar component. In the interaction studies reported in this paper both albumin preparations were indistinguishable.

Sodium salts of the following buffer ions were used in appropriate pH ranges; glycinate, acetate, mono- and di-basic phosphates and diethylbarbiturate (veronal). The pH of each buffer was measured with a glass electrode at 20°. Buffer ionic strengths are reported as l. mole^{-1}. To minimize specific ion effects on either the albumin–polyelectrolyte interactions (Noguchi, 1960) or the electrophoretic behaviour of the macromolecules, all buffer systems contained sodium chloride. In most cases the buffer salt contributed 0·05 to the ionic strength, the remainder being contributed by sodium chloride. Buffer conductivities were measured at 0°, with a Mullard conductivity bridge. All mobilities reported refer to this temperature.

Methods

Electrophoresis experiments were performed with a free-boundary Tiselius apparatus manufactured by Hilger and Watts Ltd., London. The instrument was equipped with a cylindrical lens, schlieren optical system employing a half-plane diagonal diaphragm (Philpot, 1938). The diaphragm was fixed at an angle of 25° throughout the course of this study. Standard 11 ml. cells were used. In addition to photographs of the starting boundary, at least two photographs of both the descending and ascending cell limbs were taken during the course of an experiment. Measurements of the mobilities and areas were made from enlarged ($\times 2{\cdot}5$) tracings of the photographs. Unless specifically stated otherwise, all mobilities were calculated from descending boundary data.

Solutions were prepared by adding a measured volume of buffer to weighed amounts of bovine plasma albumin and the dextran derivative. Concentrations of bovine plasma albumin and the dextran derivatives are reported as g./100 ml. of solvent. In preparation for an electrophoresis experiment, 15 ml. of solution was dialysed against 1·5 l. of buffer with stirring at 4·0° for 16–20 hr. Because of the difficulty in estimating the concentration of either the dextran amine or the dextran sulphate in the presence of protein, no attempt was made to determine the concentrations of the interactants after dialysis. However, tests made by determining bovine plasma albumin concentration spectrophotometrically before and after dialysis indicated that no significant concentration change occurred provided that the cellophan bag was tightly tied and carefully emptied after dialysis.

RESULTS

Examination of separate interactants

In order to characterize the electrophoretic behaviour and to establish the relative refractive-index increments of the individual interactants, experiments were carried out on the single components over the range of pH, concentration and ionic strength employed in the interaction studies.

The descending limb mobility of bovine plasma albumin at I 0·10, 0°, as a function of pH, is plotted in Fig. 1. Results obtained in this study are shown and, for comparison, literature values obtained under similar, but not identical, conditions are included.

Measurements carried out at I 0·05 and 0·20, at selected pH values, indicated that the mobility of bovine plasma albumin was essentially independent of ionic strength over this range in the buffer systems employed.

Table 1. *Properties of dextran derivatives*

Preparation	Sulphur (%)	Nitrogen (%)	$[\eta]$ of parent dextran	Mean mol. wt. of derivative	No. of charges per glucose unit
Dextran sulphate E-3	18·45	—	0·10	$2{\cdot}7 \times 10^4$	2·3
Dextran sulphate D-6	18·60	—	0·32	$2{\cdot}7 \times 10^5$	2·3
Dextran amine BD-9	—	4·0	0·10	$1{\cdot}6 \times 10^4$	0·8
Dextran amine BD-2	—	3·3	0·32	$1{\cdot}5 \times 10^5$	0·6

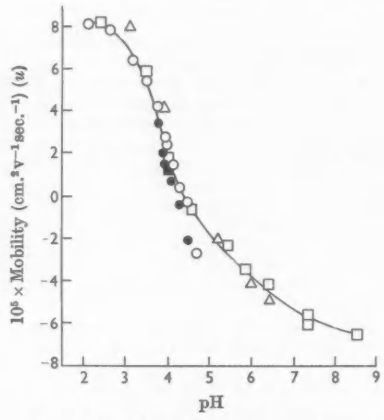

Fig. 1. Electrophoretic mobility of bovine plasma albumin (Armour crystalline). △, Singer & Campbell (1955*b*), mobility referred to 1·3°, I 0·1; ○, ●, Aoki & Foster (1956), mobility referred to 0°, I 0·10; □, ■, this paper, mobility referred to 0°, I 0·10. ○, ● and □, ■ designate the mobilities of the two components observed in the range pH 3·5–4·5.

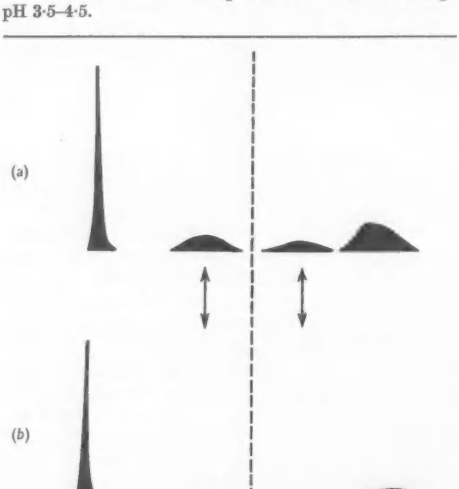

Fig. 2. Electrophoresis schlieren patterns. The vertical arrows mark the positions of the initial boundaries. (*a*) Dextran sulphate E-3, 1·50 g./100 ml., pH 7·35, I 0·10; field strength, 2·89 v cm.$^{-1}$, 81 min.; u, $-12{\cdot}0 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ (*b*) Dextran amine BD-2, 1·00 g./100 ml., pH 3·45, I 0·10; field strength, 4·52 v cm.$^{-1}$, 85 min.; u, $+8{\cdot}0 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$.

The relative refractive-index increment, expressed in arbitrary area units/unit concentration of bovine plasma albumin, was determined from the average of the total areas under the schlieren curves in both cell limbs, and was found to be 277 ± 4. Within the limits of error of the determination, the relative refractive-index increments were found to be independent of bovine plasma albumin concentration, ionic strength and pH over the range of conditions employed in this study.

The electrophoretic behaviour of the dextran sulphates was investigated in the range from pH 5·4 to 8·5 and at I 0·10 and 0·20. The two dextran sulphate preparations behaved very similarly under these conditions. Each preparation migrated as a single asymmetric peak which had a weight-average mobility, for the E-3 preparation, of $(-11{\cdot}9 \pm 0{\cdot}2) \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$, and for the D-6 preparation $(-11{\cdot}3 \pm 0{\cdot}2) \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$. The mobility was independent of the pH and ionic strength, but the shapes of both descending and ascending boundaries were dependent to some extent upon these two parameters. This dependence probably reflects interactions between the dextran sulphate and the specific buffer ions. Schlieren patterns are shown in Fig. 2*a* for the E-3 dextran sulphate at pH 7·35, I 0·20.

The number of squares of area/unit concentration for the E-3 dextran sulphate was found to be 166 ± 3, and, for the D-6 preparation, 159 ± 3. Under the conditions employed in this study the relative refractive-index increments proved to be independent of pH, ionic strength and dextran sulphate concentration.

Exhaustive dialysis at 4° of a concentrated dextran sulphate solution against a limited volume of buffer indicated that if dialysable components were present they represented less than 3% of the total dextran sulphate. In these tests the concentration of dextran sulphate outside the dialysis bag was estimated by means of the metachromasia exhibited by toluidine blue in the presence of dextran sulphate (Walton & Ricketts, 1954).

Both dextran amines gave rise to a single moving boundary, which was very slightly skewed. The weight-average mobility was independent of pH over the range from 2·5 to 4·5 and independent of ionic strength in the interval from 0·05 to 0·20. The mobilities determined from descending limb data were: BD-9 $(8{\cdot}18 \pm 0{\cdot}3) \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$, and BD-2 $(8{\cdot}05 \pm 0{\cdot}3) \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$. For the BD-9 preparation the number of squares of area/unit concentration of dextran amine was 239 ± 2, and for BD-2 preparation, 244 ± 2. Both of these values, within the limits of error, proved to be independent of concentration, pH and ionic strength. Schlieren patterns of BD-2 at pH 3·45, I 0·10, are shown in Fig. 2*b*.

Interaction studies

Systems containing both bovine plasma albumin and dextran sulphate as solute components were examined electrophoretically in the pH interval from 5·4 to 8·5 for evidence of interactions between the macromolecular species. A parallel investigation was carried out with mixtures of bovine plasma albumin and dextran amine in the range pH 2·4–4·6.

As an internal check on the composition of each solution and on the planimetry, the average of the measured total ascending and total descending areas (including anomalies) was compared with the expected total area calculated from the refractometric data and known concentrations of interactants. In the interaction studies with dextran sulphate the difference between total measured area and total calculated area averaged over all experiments was 2·7 %, with a maximum difference of 6·2 %. The error varied from 0 to 3·8 % with a mean error of 1·8 % in the interaction studies involving dextran amine. Formation of insoluble interaction products was not observed under any set of conditions employed in this work.

Electrophoretic evidence for interaction between macromolecular solutes is obtained if the schlieren patterns of the moving-boundary systems in one or both cell limbs do not represent the simple sum of the corresponding patterns obtained upon electrophoresis of the individual components under similar conditions (Alberty, 1953). Strict additivity is not observed even if no interaction between solutes occurs. These deviations are usually small compared with the deviations resulting from interaction and are discussed below.

When non-additivity of the system is attributed exclusively to solute interaction, a convenient empirical measure of the 'degree of interaction' (α) is defined by

$$\alpha = 1 - F_2/f_2. \qquad (1)$$

Here F_2 is the apparent refractometric fraction of free interactant comprising the slowest moving boundary in the limb, and f_2 is the true refractometric fraction of this interactant in the system.

Although in this paper only values of α will be reported, an analogous measure of interaction, $\beta = 1 - F_1/f_1$, based on the apparent refractometric fraction of dextran derivative in the fastest migrating boundary in the ascending limb, can also be defined. The subscripts 1 and 2 designate the dextran derivative and bovine plasma albumin respectively.

The apparent refractometric fraction, F_i, is defined by

$$F_i = A_i/\Sigma A_i. \qquad (2)$$

Here A_i is the area in arbitrary units under the schlieren curve of the ith moving boundary and ΣA_i is the total area under all moving boundaries in the cell limb. The true refractometric fraction f_i is given by

$$f_i = R_i C_i/\Sigma R_i C_i. \qquad (3)$$

Here R_i denotes the relative refractive-index increment of the ith interactant measured in arbitrary area units per unit concentration. C_i is the concentration of this interactant.

In all systems studied the mobility of bovine plasma albumin was less than the mobility of the dextran derivative and thus less than the mobility of any complex species. Therefore free bovine plasma albumin in the equilibrium mixture and free bovine plasma albumin produced by re-equilibration within the moving-boundary system should appear as the slowest moving boundary in the descending limb. If interaction occurs between the bovine plasma albumin and the dextran derivative, the apparent refractometric fraction of bovine plasma albumin across this slowest descending boundary will be less than the known true refractometric fraction of bovine plasma albumin in the system. This will be the case whether re-equilibration occurs within the boundaries or not. Thus if no interaction occurs, $\alpha = 0$, but if all bovine plasma albumin is held in complex and no re-equilibration occurs, then $\alpha = 1$. It is evident that α is a complicated function of the concentration of interactants, the number of complex species and the rates of complex dissociation and formation. It is also evident that if re-equilibration occurs, then α cannot be related simply to the concentration of free bovine plasma albumin in the equilibrium mixture.

If α is to be a measure of interaction, then it is necessary that the slowest descending boundary be entirely free of the dextran derivative, i.e. that it be composed of bovine plasma albumin only. Similarly, if β is to be a measure of interaction, then the fastest ascending boundary must be free of bovine plasma albumin. A series of isolation experiments carried out with a split cell (Tiselius, 1937) indicated that these conditions obtained. (The design of this type of cell makes it possible to isolate the slowest moving component in the descending limb, and the fastest moving component in the ascending limb.) A summary of the results obtained in two typical isolation experiments is given in Table 2.

The results of the studies of the bovine plasma albumin–dextran sulphate interaction are presented in Tables 3–5. These Tables list the conditions of each experiment, the true refractometric fraction f_2 and the apparent descending limb refractometric fraction F_2 for bovine plasma albumin, the measured value of α, and the calculated value of α referred to in the Discussion and the mobilities of the fast boundary and the bovine plasma albumin boundary.

Table 3 illustrates the dependence of the measured α on pH. α, essentially zero at pH 8·50, rises

Table 2. *Results of a typical isolation experiment*

BPA, Bovine plasma albumin.

Expt. no.	Composition of buffer	pH	I	Composition of equil. solution: BPA (g./100 ml.)	Composition of equil. solution: E-3 (g./100 ml.)	Composition of isolated fraction: BPA* (g./100 ml.)	Composition of isolated fraction: E-3† (g./100 ml.)	Cell limb
41	Phosphate–NaCl	7·32	0·10	1·00	1·50	0·263	<0·002	Desc.
42	Phosphate–NaCl	7·30	0·10	1·53	1·01	<0·005	0·23	Asc.

* BPA concentrations determined spectrophotometrically at 280 mμ, $E^{1\%}_{1\,cm.}$ 6·55.
† E-3 concentration determined by fibrinogen-clotting assay.

Table 3. *Bovine plasma albumin–dextran sulphate interaction as a function of* pH

Ionic strength 0·10. Bovine plasma albumin (BPA) lot BA-0170. Descending cell limb.

Expt. no.	pH (20°)	Dextran sulphate (g./100 ml.): E-3	Dextran sulphate (g./100 ml.): D-6	BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	$-10^5 u$ (cm.²v⁻¹ sec.⁻¹): Fast	$-10^5 u$ (cm.²v⁻¹ sec.⁻¹): BPA
44	8·50	0·84	—	1·50	0·738	0·756	−0·02	0·068	10·6	5·62
15	7·40	0·42	—	1·52	0·850	0·616	0·28	0·032	12·1	4·80
26	6·40	0·43	—	1·50	0·847	0·311	0·63	0·011	9·7	4·23
45	8·50	—	0·98	1·53	0·720	0·740	−0·02	0·092	10·6	5·63
39	7·35	—	0·49	1·50	0·835	0·636	0·24	0·048	11·5	4·53
42	6·40	—	0·51	1·50	0·829	0·382	0·54	0·018	8·5	3·66
68	5·85	—	0·51	1·41*	0·832	0·151	0·82	<0·01	7·15	3·47
70	5·40	—	0·53	1·49*	0·831	0	1·00	<0·01	5·90	—

* BPA lot BE-1370.

Table 4. *Bovine plasma albumin–dextran sulphate interaction as a function of ionic strength*

Bovine plasma albumin (BPA) lot BA-0170. Descending cell limb.

Expt. no.	pH (20°)	I (l.mole⁻¹)	E-3 (g./100 ml.)	BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	$-10^5 u$ (cm.²v⁻¹ sec.⁻¹): Fast	$-10^5 u$ (cm.²v⁻¹ sec.⁻¹): BPA
15	7·40	0·10	0·42	1·52	0·850	0·616	0·28	0·032	12·1	4·80
13	7·40	0·10	1·26	1·52	0·656	0·381	0·42	0·062	12·0	4·10
23	7·35	0·20	0·42	1·51	0·850	0·834	0·02	0·012	12·2	4·71
22	7·35	0·20	1·24	1·51	0·658	0·634	0·04	0·034	11·7	4·15

Table 5. *Bovine plasma albumin–dextran sulphate interaction as a function of interactant concentrations*

Ionic strength 0·10. Bovine plasma albumin (BPA) lot BA-0170. Descending cell limb.

Expt. no.	pH (20°)	Dextran sulphate (g./100 ml.): E-3	Dextran sulphate (g./100 ml.): D-6	BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	$-10^5 u$ (cm.²v⁻¹ sec.⁻¹): Fast	$-10^5 u$ (cm.²v⁻¹ sec.⁻¹): BPA
85	7·35	0·43	—	0·52*	0·668	0·505	0·24	0·018	13·6	4·90
84	7·35	0·44	—	1·00*	0·789	0·640	0·19	0·022	13·3	5·00
15	7·40	0·42	—	1·52	0·850	0·616	0·28	0·032	12·1	4·80
29	7·35	0·84	—	1·49	0·740	0·484	0·35	0·058	12·2	4·40
51	7·35	1·27	—	0·20	0·197	0·132	0·33	0·041	11·5	3·80
34	7·32	1·27	—	0·50	0·383	0·231	0·40	0·058	12·3	4·07
33	7·32	1·27	—	1·01	0·557	0·342	0·38	0·072	11·9	4·00
13	7·40	1·26	—	1·52	0·656	0·381	0·42	0·062	12·0	4·10
26	6·40	0·43	—	1·50	0·847	0·311	0·63	0·011	9·7	4·23
25	6·40	1·24	—	1·50	0·656	0·264	0·60	<0·01	10·5	3·50
77	5·85	—	0·18	1·42*	0·934	0·434	0·53	<0·01	5·20	3·31
68	5·85	—	0·50	1·41*	0·832	0·151	0·82	<0·01	7·15	3·47
76	5·40	—	0·19	1·42*	0·929	0·073	0·92	<0·01	3·53	2·33
70	5·40	—	0·53	1·49*	0·831	0	1·00	<0·01	5·90	—

* BPA lot BE-1370.

steadily to the maximum value of 1·0 at pH 5·40. The electrophoresis patterns obtained at pH 8·50 were almost exactly the sum of schlieren patterns obtained for the individual interactants. At pH 7·4 two moving boundaries were evident in the descending limb, but three moving boundaries developed in the ascending limb. However, at pH 6·40 the number of boundaries in the two limbs were reversed; three developed in the descending limb, and two developed in the ascending limb. At pH 5·85 only one ascending boundary developed, and this boundary migrated with a mobility about one-half of that which would be expected for free dextran sulphate. In the descending limb two boundaries were observed at this pH. At pH 5·40 only one moving boundary was observed in each cell limb. The descending boundary at this pH had a mobility midway between the mobilities of D-6 and bovine plasma albumin. Except for certain details in the ascending patterns E-3 and D-6 were indistinguishable in their interaction with bovine plasma albumin. The schlieren patterns of ascending and descending cell limbs for five conditions of interaction are reproduced in Fig. 3.

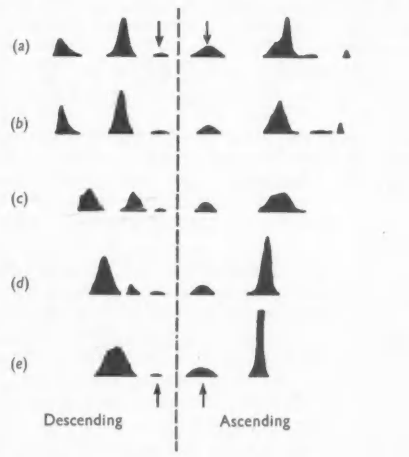

Fig. 3. Electrophoresis schlieren patterns illustrating the interactions between bovine plasma albumin and dextran sulphate as a function of pH at I 0·10. The vertical arrows mark the positions of the initial boundaries. (*a*) pH 7·4, 4·84 v cm.$^{-1}$; bovine plasma albumin, 1·52 g./100 ml.; E-3, 0·42 g./100 ml.; 85 min. (*b*) pH 7·3, 4·72 v cm.$^{-1}$; bovine plasma albumin, 1·50 g./100 ml.; D-6, 0·49 g./100 ml.; 90 min. (*c*) pH 6·40, 6·25 v cm.$^{-1}$; bovine plasma albumin, 1·50 g./100 ml.; D-6, 0·51 g./100 ml.; 70 min. (*d*) pH 5·85, 5·99 v cm.$^{-1}$; bovine plasma albumin, 1·41 g./100 ml.; D-6, 0·50 g./100 ml.; 65 min. (*e*) pH 5·40, 5·58 v cm.$^{-1}$; bovine plasma albumin, 1·49 g./100 ml.; D-6, 0·53 g./100 ml.; 65 min.

Table 4 summarizes the results of four experiments carried out at pH 7·4, but at two different ionic strengths (I 0·10 and 0·20). It appears from this limited set of data that the interaction decreases sharply with increasing ionic strength. The schlieren patterns obtained at I 0·20 are very closely the sum of the schlieren patterns obtained under these conditions for the individual interactants.

The variation of the degree of interaction with concentration of interactant is illustrated by the data assembled in Table 5.

Tables 6 and 7 summarize the electrophoresis results for the bovine plasma albumin–dextran amine systems.

DISCUSSION

Two types of deviations might be expected in systems containing two interacting solute components: (*a*) the number of moving boundaries in one or both cell limbs is either greater or less than two; (*b*) the apparent descending limb refractometric fraction of the slower-moving interactant is less than the true refractometric fraction, and the apparent ascending limb refractometric fraction of the faster-migrating interactant is less than the true refractometric fractions. It is important to examine these assumptions to determine whether observed deviations from simple additivity can in fact be attributed solely to macromolecular interaction in the systems investigated.

Observed deviations of type (*a*) can normally only be attributed to interaction between solutes. Consequently, deviations of this type constitute the strongest evidence for the existence of such interactions. It must be pointed out, however, that in buffer solvents of the type used in this work (composed of the buffer plus sodium chloride) a rapidly moving boundary which does not correspond to a macromolecular component might possibly be observed. In the buffer system used above pH 3·5, such a boundary would be expected to have a mobility between that of Cl^- ion and the buffer anion (Svensson, 1946). Since in these systems the mobilities of the Cl^- ion and the buffer anion were greater than the mobility of the dextran derivative, the mobility of a spurious boundary would be greater than the mobility of the dextran derivative boundary. In no experiment was a boundary observed to have a mobility greater than that of the dextran derivative.

With the glycine–sodium chloride buffer systems used at pH 2·4 and 3·5, a spurious boundary would be expected to have a mobility less than that of the dextran amine. However, in the experiments in these buffers only two moving boundaries were observed. Therefore in the experiments in which more than two moving boundaries were observed the existence of additional boundaries must

Table 6. *Bovine plasma albumin–dextran amine interaction as a function of* pH

Ionic strength 0·10. Bovine plasma albumin (BPA) lot BA-0170. Descending cell limb.

Expt. no.	pH (20°)	Dextran amine (g./100 ml.) BD-9	BD-2	BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	10^5u (cm.^{2}v^{-1} sec.$^{-1}$) Fast	BPA
72	2·40	1·01	—	0·98*	0·530	0·484	0·09	—	8·15	7·97
66	3·50	1·00	—	1·00*	0·537	0·478	0·11	0·13	8·08	4·72
54	4·05	1·02	—	1·02	0·524	0·473	0·10	0·07	8·51	0·82
56	4·55	1·01	—	0·99	0·519	0·526	−0·01	−0·01	8·44	−0·86
73	2·40	—	1·00	1·01*	0·534	0·353	0·34	—	8·10	7·80
40	3·50	—	1·00	1·00	0·518	0·422	0·18	0·23	8·06	4·92
75	4·55	—	1·00	1·01*	0·534	0·532	0	−0·01	7·97	−0·90

* BPA lot BE-1370.

Table 7. *Bovine plasma albumin–dextran amine interaction as a function of ionic strength and interactant concentration*

pH 3·50. Descending cell limb. BPA, Bovine plasma albumin.

Expt. no.	I (l.mole^{-1})	BD-2 (g./100 ml.)	BPA (g./100 ml.)	f_2	F_2	α (measured)	α (calculated)	10^5u (cm.^{2}v^{-1} sec.$^{-1}$) Fast	BPA
48	0·05	0·50	1·50	0·764	0·696	0·09	—	7·80	5·00
49	0·05	1·50	0·52	0·271	0·231	0·15	—	7·46	3·75
18	0·05	1·50	1·51	0·519	0·416	0·20	—	7·27	3·76
37	0·10	0·49	1·01	0·689	0·630	0·08	0·11	8·05	5·38
40	0·10	1·00	1·00	0·518	0·422	0·18	0·23	8·06	4·92
47	0·10	1·51	1·00	0·415	0·324	0·22	0·25	7·50	4·18
21	0·20	0·50	1·02	0·686	0·647	0·06	0·06	8·00	5·60
20	0·20	1·00	0·50	0·349	0·351	0	0·10	7·90	5·60
30	0·20	1·01	1·02	0·519	0·532	−0·02	0·06	7·80	5·45

be attributed to interaction. In the bovine plasma albumin–dextran sulphate experiments deviations of type (*a*) were observed in all systems having values of $\alpha > 0{\cdot}10$. However, in the bovine plasma albumin–dextran amine systems evidence of this kind for interaction was not obtained since two ascending and two descending moving boundaries were observed in all experiments.

While deviations of type (*b*) would be expected to occur in interacting systems, the observation of this sort of deviation does not by itself prove macromolecular interaction. The apparent and true refractometric fractions cannot, in general, be expected to be equal, even in simple systems in which there is no interaction between components. Deviations in the apparent refractometric fractions in a non-interacting system are for the most part due to the fact that small gradients of components which do not disappear in a given moving boundary are superimposed on the gradient of the component which does disappear. As a result, the apparent refractometric fractions have different values in the ascending and descending limbs, and are functions of the ratio of total protein concentration to buffer salt concentration (Svensson, 1943; Armstrong, Budka & Morrison, 1947; Longsworth, 1947; Nichol & Gosting, 1958). In many protein systems the deviations in apparent refractometric fractions seem to decrease as this ratio is decreased. Also, the deviations are less in systems in which large differences exist between the mobilities of the protein components (Svensson, Benjaminsson & Brattsten, 1949).

Quantitative estimates of the magnitude of type (*b*) deviations expected in the absence of interaction cannot be made from the moving-boundary theory for weak electrolytes (Svensson, 1948; Alberty & Nichol, 1948; Alberty, 1950). However, several authors (Longsworth, 1947, 1959; Armstrong *et al.* 1947; Alberty, 1948; Cann, 1949; Brown & Timasheff, 1959) have utilized the moving-boundary theory for strong electrolytes (Dole, 1945; Svensson, 1946) with considerable success in discussing electrophoretic behaviour in protein systems. The series solutions for the Dole equations recently obtained by Nichol & Gosting (1958) have considerably simplified the calculation of apparent refractometric fractions with the theory for strong electrolytes. These equations have been used to calculate the values of α which would be expected in the absence of interaction in the bovine plasma albumin–dextran derivative systems studied.

Although, in principle, apparent refractometric fractions can be calculated following Nichol &

Gosting (1958) for systems containing more than one supporting electrolyte, the calculations are very cumbersome. In order to simplify the calculations all ions other than the macromolecular ions in the systems studied have been taken to be K^+ and Cl^- ions. It is not likely that this simplification is the source of any significant error in the calculation. To carry out these calculations it is necessary to know the equivalent concentrations and the equivalent refractions of both the bovine plasma albumin and the dextran derivative under the conditions of a particular experiment. The equivalent concentration C_i of the ith component is given by the following relation:

$$C_i = 10p_i\left[\frac{Z_i}{M_i}\right]. \quad (4)$$

Here p_i is the concentration (g./100 ml. of soln.), Z_i is the net charge per mole in protonic units and M_i is the molecular weight of the ith component. The equivalent refraction k_i may be obtained from the specific refraction k_i for the ith component by

$$k_i = \frac{k_i}{10}\left[\frac{M_i}{Z_i}\right]. \quad (5)$$

For the bovine plasma albumin at pH 7·4 a net charge of -22 was estimated from the titration data of Tanford, Swanson & Shore (1955), with allowance being made for Cl^- ion-binding. The effective net charge on the bovine plasma albumin at some other pH was then assumed to be proportional to the electrophoretic mobility at that pH (Brown & Timasheff, 1959). The molecular weight of the bovine plasma albumin was taken to be $6{\cdot}9 \times 10^4$ and specific refraction was taken as 0·00186 $(\text{g. dl.}^{-1})^{-1}$ (Armstrong *et al.* 1947).

The charge Z appearing in equations (4) and (5) is the charge associated with the electrokinetic unit in solution. Because a portion of the gegen-ion atmosphere migrates with the macromolecule, the effective charge of the electrokinetic unit will be less than the net charge on the macromolecular ion as estimated from titration data. With the bovine plasma albumin, this difference is probably small because of the relatively small net charge on the protein, and no significant error results from estimating Z on the basis of titration data. However, such cannot be expected to be the case with the dextran sulphates and dextran amines. Polyelectrolytes of charge densities comparable with the dextran derivatives have been shown to bind as much as 90% of the gegen-ion atmosphere into the kinetic unit (Huizenga, Grieger & Wall, 1950; Mathieson & McLaren, 1956; Botré, Crescenzi, Liquori & Mele, 1959). Clearly the effective charge on the dextran derivatives cannot be calculated from the sulphur or nitrogen content. For the purpose of the calculations based on the Nichol–Gosting equations, and in the absence of more detailed information, it has been assumed that the charge/mass ratio (Z/M) of the dextran derivative is proportional to the electrophoretic mobility, and that the proportionality constant is the same for both bovine plasma albumin and the dextran derivative. The value of Z/M for the dextran derivative was then estimated from the following equation:

$$\left[\frac{Z}{M}\right]_1 = \frac{u_1}{u_2}\left[\frac{Z}{M}\right]_2. \quad (6)$$

Here u is mobility, and the subscripts 1 and 2 refer to the dextran derivative and bovine plasma albumin respectively. The values of $(Z/M)_2$ and u_2 used were those obtained at pH 7·4. Values of equivalent refractions for the dextran derivatives were calculated from the relative refractive-index increments for the derivatives and for bovine plasma albumin given in the preceding section and from the specific refraction for bovine plasma albumin.

The calculated values of α are listed together with the measured values of α in Tables 3–7. For the bovine plasma albumin–dextran sulphate systems (Tables 3–5) the calculated values of α are less than 0·10 in all cases. Under these conditions no evidence of type (*a*) for interaction was obtained; the calculated and measured values of α are in good agreement (pH 7·4, I 0·20 and pH 8·5, I 0·10). However, in cases where evidence of type (*a*) interaction was obtained, the measured values of α are at least six times as great as the calculated values.

In the bovine plasma albumin–dextran amine systems there was no evidence of type (*a*) to suggest the existence of interactions. In addition, comparison of the measured and calculated values of α shows good agreement between the two sets of values. This suggests that essentially no interaction occurs in these systems.

However, evidence of a somewhat different nature was obtained for interaction between bovine plasma albumin and BD-9 at pH 4·05, I 0·10. Under these conditions in a system containing both bovine plasma albumin and BD-9 the slow descending boundary composed only of bovine plasma albumin failed to split in two, as is the case with pure bovine plasma albumin (Aoki & Foster, 1956). Substitution of the parent dextran, from which BD-9 was prepared, for BD-9 in a similar experiment, however, did not prevent formation of the expected double BPA boundary. These observations are illustrated in Fig. 4. It is evident from these experiments that an interaction of some sort occurs between bovine plasma albumin and BD-9 under these conditions. The interaction appears to affect directly the bovine plasma albumin-isomerization reaction. The fact that the two albumin species were not observed in the slow descending boundary

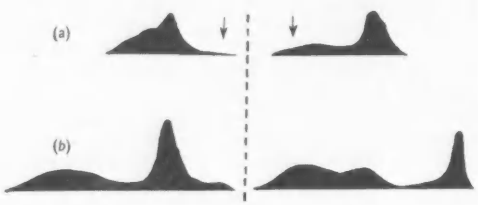

Fig. 4. Electrophoresis schlieren patterns obtained at pH 4·05, I 0·10. The vertical arrows mark the positions of the initial boundaries. (*a*) Bovine plasma albumin, 1·00 g./100 ml.; field strength 5·23 v cm.$^{-1}$, 120 min. (*b*) Bovine plasma albumin, 1·00 g./100 ml. + dextran amine BD-9, 1·00 g./100 ml.; field strength 5·23 v cm.$^{-1}$, 120 min. (*c*) Bovine plasma albumin, 1·00 g./100 ml. + dextran, 1·00 g./100 ml.; field strength 5·23 v cm.$^{-1}$, 150 min.

which is free of BD-9 (at least within the limit of analysis) suggests that the interaction produces an irreversible alteration in the bovine plasma albumin.

Longsworth & MacInnes (1942) investigated the interaction between ovalbumin (P) and nucleic acid (N) and described the electrophoretic behaviour which might be expected in five cases of complex formation based on the reaction:

$$P + rN \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PN_r. \qquad (7)$$

In this equation k_1 and k_{-1} are the rate constants for the forward and reverse reaction respectively and r is the number of moles of nucleic acid reacting with 1 mole of protein.

The electrophoretic behaviour observed in the present research could be expected to be similar to two of the cases described by Longsworth & MacInnes. In the fourth case the rate constants k_1 and k_{-1} are of such magnitude that the rate of adjustment of the equilibrium is comparable with the rate of electrophoretic separation of the components. In this system, however, the mobilities and areas of the components would be time-dependent and in no instance did the data reported in this paper show such a time-dependence. Thus it would appear that the behaviour of the present system is only directly analogous to the fifth case described by Longsworth & MacInnes. In this case the rate constants k_1 and k_{-1} are large and of the same order of magnitude, with the result that the equilibrium is adjusted within the moving-boundary system as rapidly as is required by the electrophoretic separation of components. The migration of the faster interactant into solvent in the ascending limb, but into solution in the descending limb, results in the separation of pure fast interactant as the leading boundary in the ascending limb and pure slow interactant as the trailing boundary in the descending limb. Because of interaction the apparent refractometric fractions determined from these two boundaries will be less than the true refractometric fractions of the corresponding interactants. These apparent fractions are, however, larger than would be the case if re-equilibration did not take place, due to the dissociation in each limb of part of the complex. As a direct consequence of re-equilibration there is no boundary which may be identified simply with the complex, and, in addition, the total number of moving boundaries in each limb may not be equal and may be less than, equal to or greater than two. A simple interpretation of the apparent refractometric fractions will therefore not yield the true equilibrium constant for complex formation.

No general theory is available for the detailed interpretation of moving-boundary patterns in interacting systems exhibiting re-equilibration. Theoretical treatments for certain special cases of complex formation have been presented (Smith & Briggs, 1950; Alberty & Marvin, 1950; Field & Ogston, 1955; Gilbert & Jenkins, 1956, 1959; Cann, Kirkwood & Brown, 1957; Kegeles & Rao, 1958; Gilbert, 1959).

The theory of Gilbert & Jenkins is based on the reaction given by equation (7), when $r = 1$. Although the bovine plasma albumin–dextran derivative interactions can in general be expected to be more complicated than this, it does appear that under one set of conditions the bovine plasma albumin–E-3 system (pH 7·4, I 0·10) behaved approximately like one of the systems discussed by Gilbert & Jenkins (1959), in which the complex and the faster interactant have the same mobility; three moving boundaries are predicted for the ascending limb and two for the descending limb. In addition, the concentration of the slower interactant across the trailing descending boundary is shown to be the concentration of this component in the bulk equilibrium mixture. It is therefore possible to calculate the equilibrium constant for complex formation from the descending limb data. Gilbert & Jenkins (1959) also predict for the ascending limb data that a plot of $1/(u_c - u_2)$ against, the reciprocal of total concentration of fast interactant (component 1), should be linear with an intercept equal to $1/(u_1 - u_2)$. Here u_c is the mobility of the second

boundary in the ascending limb, u_1 and u_2 are the mobilities of the fast and slow interactants respectively.

The bovine plasma albumin–E3 system at pH 7·4, I 0·10, did exhibit at all concentrations of interactants three ascending and two descending moving boundaries. In addition, the mobility of the fast descending boundary was equal to that of E-3 (Table 3). This could only occur if the complex and E-3 had the same mobility. The plot suggested by Gilbert & Jenkins of the ascending limb mobility data is presented in Fig. 5. It can be seen that the data are reasonably well fitted by a straight line with intercept 0·088, in good agreement with the predicted value of 0·092. Table 8 lists the equilibrium constant calculated from the descending limb data for each of eight experiments. In these calculations the molecular weights of bovine plasma albumin and E-3 dextran sulphate were taken as $6{\cdot}9 \times 10^4$ g.mole^{-1} and $2{\cdot}7 \times 10^4$ g.mole^{-1} respectively. The equilibrium concentration of bovine plasma albumin was calculated from the apparent refractometric fraction of this component, the average total measured area and the relative refractive index increment of bovine plasma albumin. Agreement among the values of the equilibrium constant listed in Table 8 is fairly good. It must be noted in this regard that no allowance has been made for the presence of buffer ion and sodium chloride gradients within the moving boundaries. In addition to the deviations in F_2 which have been discussed, it would be expected that these gradients would effect re-equilibration at the moving boundaries, since the interaction is a function of both pH and ionic strength.

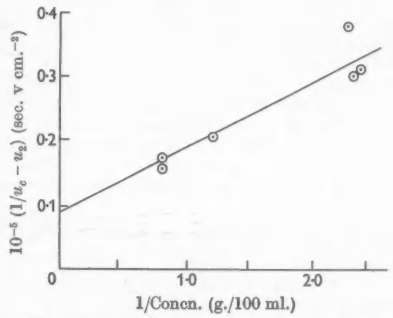

Fig. 5. Plot of the ascending mobility function of Gilbert & Jenkins (1959) against the reciprocal of the total concentration of dextran sulphate (fast interactant). The system consists of dextran sulphate, E-3 (interactant 1) and bovine plasma albumin (interactant 2), pH 7·4, I 0·10.

The fact that the interaction between bovine plasma albumin and the dextran sulphates is a function of the ionic strength suggests that the primary force involved is electrostatic. Furthermore, it would seem that electrostatic-attraction forces must be involved in stabilizing the complex, since interaction decreases with increasing ionic strength. These observations suggest that the bonds involved in complex formation are between the negative charges on the dextran sulphate and the residual positive charges on the bovine plasma albumin (cf. Haurowitz, 1950; Sasaki & Noguchi, 1959). It is then probable that two causes operate to produce the observed decrease in interaction at higher pH values: (1) as the pH is raised the net negative charge on the bovine plasma albumin is increased and hence the electrostatic repulsion between the protein and the dextran sulphate is increased (Noguchi, 1956); (2) as the pH is raised the number of positive charges on the protein which are available for interaction is decreased.

Exposure of bovine plasma albumin to dextran amine at pH 4·05 prevents the splitting of the bovine plasma albumin during electrophoresis at this pH, but no other evidence was obtained for bovine plasma albumin–dextran amine interaction. Soluble complexes of the type observed in the bovine plasma albumin–dextran sulphate systems certainly do not form in the bovine plasma albumin–dextran amine systems. This difference may in part be due to the fact that the charge density of the dextran amines is about one-third of

Table 8. *Equilibrium constant for bovine plasma albumin–dextran sulphate interaction*

pH 7·4, I 0·10. Calculated according to Gilbert & Jenkins (1959).

Expt. no.	E-3 (g./100 ml.)	BPA (g./100 ml.)	F_2 (desc.)	Total area in squares	BPA (g./100 ml.; desc.)	10^{-4} K (l.mole^{-1})
85	0·43	0·52*	0·505	217	0·396	2·22
13	1·26	1·52	0·381	634	0·922	1·69
29	0·84	1·49	0·484	520	0·961	2·37
15	0·42	1·52	0·616	478	1·125	3·58
33	1·27	1·01	0·342	485	0·658	1·43
34	1·27	0·50	0·231	349	0·308	1·42
51	1·27	0·20	0·132	269	0·135	1·04
84	0·44	1·00*	0·640	359	0·829	1·51
						Average 1·91

* BPA lot BE-1370.

that of the dextran sulphates (Table 1). There is, however, considerable evidence to indicate that organic cations, in general, interact much less strongly with plasma albumin than do anions (e.g. Klotz, Gelewitz & Urquhart, 1952; McKernan & Ricketts, 1960). Haurowitz (1936) has suggested that the apparent inability of serum albumin to form strong complexes with basic colloids may be explained by assuming that the CO_2^- groups are located in the interior of the protein molecule and hence are not available for interaction. Klotz *et al.* (1952) have proposed that the CO_2^- groups are unavailable for cation binding because of intramolecular interaction with the hydroxyl groups of neighbouring hydroxyamino acids. It is also possible that the detailed charge configuration on the protein surface is an important parameter in complex formation. For example, clusters of closely grouped positive charges on the bovine plasma albumin could be important in the formation of bovine plasma albumin–anion complexes; the absence of similar clusters of negative charge would then be reflected in the considerably lower affinity of bovine plasma albumin for organic cations.

SUMMARY

1. An electrophoretic investigation of the formation of soluble interaction complexes between bovine plasma albumin and charged dextran derivatives (sulphate esters and diethylaminoethyl ethers) is described. The investigation is limited to conditions of pH such that the net charge on the protein and the charge on the polyelectrolyte have the same signs.

2. In the dextran sulphate systems soluble complex formation is maximal at the isoelectric pH of the protein and decreases with increasing pH. Complexes cannot be detected above pH 8·5. Complex formation decreases with increasing ionic strength. The equilibrium constant for complex formation is calculated from the electrophoresis results under one set of conditions which constitute a special case of the theory of Gilbert & Jenkins.

3. Dextran amine is shown to suppress the albumin isomerization reaction at pH 4·0. Except for this observation, no evidence has been obtained for complex formation between the bovine plasma albumin and the dextran amines.

4. The mobility of bovine plasma albumin at I 0·10, 0°, is determined as a function of pH.

5. Deviations from simple electrophoretic behaviour expected in the absence of interaction in the systems studied are evaluated with the moving-boundary theory for strong electrolytes.

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Eugenol Lignin: its Chemical Properties and Significance

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Observations by several investigators have shown that many different substances may appear to act as precursors of lignin in growing plants or in artificial systems. Stone (1953) has pointed out that deposition of a radioactive lignin from a labelled precursor is not in itself proof that the supposed precursor has been incorporated into the lignin. The radioactivity may be due merely to co-polymerization.

Schubert, Acerbo & Nord (1957) demonstrated the incorporation of labelled *p*-hydroxyphenylpyruvic acid into the lignin of sugar cane. Acerbo, Schubert & Nord (1958) showed that the carboxyl-labelled acid gave an active lignin but that the vanillin produced from it was inactive, thus suggesting that the aromatic ring of the acid was incorporated as such into the lignin. Kratzl & Billek (1959) found that the [3-^{14}C]-labelled acid, when infused into lignifying spruce tissue, gave an active lignin but that the vanillin produced from it was of very low activity. This appears to be a case of co-polymerization. However, Brown, Wright & Neish (1959) found that the wheat plant could synthesize guaiacyl and syringyl radicals from this precursor.

Artificial lignification of *Elodea* sections in solutions containing peroxide and eugenol has been demonstrated by Siegel (1954). In a system with added peroxidase a lignin was deposited on a filter-paper matrix (Siegel, 1956). The lignin formed was similar to natural lignin in ultimate composition (Siegel, 1957). Higuchi (1957), experimenting with bamboo-shoot and horseradish enzyme preparations, observed the formation of a lignin-like substance from old, but not from freshly distilled, eugenol. He concluded that the lignin-like substance was formed from coniferaldehyde in the eugenol. On the other hand, Stafford (1960) found that a lignin-like substance was deposited in *Phleum* leaf sections even by freshly distilled eugenol. This 'lignin', although giving a positive phloroglucinol test, differed from the natural lignin or that formed from ferulic acid in not being extractable with 2% sodium hydroxide.

Artificial lignification of oat (*Avena*) coleoptiles, *Elodea* internodes and other tissues in the same medium as used by Siegel in his experiments was studied by Wardrop & Davies (1959) mainly from the morphological point of view, and they concluded from electron micrographs and staining reactions of the lignifying tissue that the artificial lignin was deposited between the cellulose microfibrils in the wall in the same way as natural lignin.

These morphological studies leave unanswered the question of whether the lignin deposited is identical with natural lignin. It has been pointed out by Freudenberg (1958) that no eugenol exists in cambial sap and that formation of artificial lignin from this substance has nothing to do with natural lignification. On the other hand, just as an organic substance may be synthesized by different routes, it is conceivable that a living plant could utilize a number of unnatural precursors to lay down a lignin indistinguishable from its natural lignin. For

eugenol this is not such a surprising proposition in view of the possibility of the first step being transformation into coniferyl alcohol or coniferaldehyde, or both, as suggested by Adler (1957).

The present work was undertaken primarily to determine if eugenol lignin could be distinguished chemically from natural lignin, and if so in what way it differed.

EXPERIMENTAL

Analysis and purification of eugenol. Wardrop & Davies (1959) used the phloroglucinol test for lignin in their experiments with coleoptiles. In the present work the eugenol (W. J. Bush and Co., Mentone, Victoria, Australia) was tested for phloroglucinol reaction before and after purification by washing with 20% (w/v) $NaHSO_3$ solution, followed by water-washing and distillation. Quantitative analyses of coniferaldehyde and coniferyl alcohol present in the original and purified eugenol were made by u.v.-absorption measurements as follows. The absorption curves of both the stock and purified eugenol in the range 240–400 mμ were determined by using a 0·005% solution in methanol and in methanolic KOH (0·1 N), and the $\Delta\epsilon$ curves derived. $\Delta\epsilon$ was close to zero in the range 320–400 mμ. More concentrated solutions (approx. 0·1%) were then taken and $\Delta\epsilon$ was measured at 320 and 400 mμ. The percentages of coniferaldehyde and coniferyl alcohol were then calculated with $\Delta\epsilon_{400\,m\mu}$ 32 000 for coniferaldehyde and $\Delta\epsilon_{320\,m\mu}$ 10 000 for coniferyl alcohol, these values being taken from the curves of Aulin-Erdtman (1953, 1957). Purified eugenol was used throughout the experiments described below.

Preparation of artificially lignified tissues. Lignified oat-coleoptile sections were prepared as described by Wardrop & Davies (1959) with the buffered eugenol solution described by Solberg & Higginbotham (1957), i.e. 4 m-moles of eugenol, 15 ml. of ethanol, 4 m-moles of H_2O_2 and 5 m-moles of KH_2PO_4 in 1 l. of water. In this way it was possible to prepare artificially lignified tissue in small quantities that were sufficient to permit chemical investigation on a limited scale only. The preparation with coleoptiles was extremely tedious and was discarded in favour of potato parenchyma, which could be prepared on a large scale.

Artificially lignified potato parenchyma was prepared as follows. Potatoes (9 kg.) were carefully peeled and all traces of shoots removed. Slices were treated in a Waring Blendor and the resulting pulp was allowed to stand in water (20 l.) until the starch settled to the bottom, when the suspension of pulp was poured off the starch. At this stage addition of eugenol solution to a test portion of the suspension resulted in strong yellowing of the solution presumably caused by the presence of dissolved peroxidase. After four washings, addition of eugenol solution to a test portion caused a barely perceptible yellowing of the solution. The suspended parenchyma was filtered on a Buchner filter and pressed as dry as possible.

The filter cake was broken up and stirred into 20 l. of the eugenol solution described above. After a few minutes the cellular material became a bright yellow but this faded to a dull brown within an hour. After several hours the supernatant liquor was poured off and the treatment repeated, a total of six treatments being given over a week. The preparation was then washed with water (20 l.) four times by decantation and then eight times with warm (50°) ethanol (5 l.). It was then sucked dry on a Buchner filter, washed with water and spread out to air-dry. This yielded 336 g. of material containing 15% of moisture.

Determination of lignin in artificially lignified tissues. Klason lignin was determined by adapting the procedure of Ritter, Seborg & Mitchell (1932). The tissue was stirred for 2 hr. with 72% (w/w) H_2SO_4, diluted to 3% (w/v) and boiled for 2 hr. The insoluble residue was collected by filtration on a Norton alundum crucible R.A. 360, washed, dried, weighed and analysed for methoxyl groups and nitrogen. The lignin percentage was corrected on a basis of 16% of methoxyl groups.

An estimate of the lignin present was also made by a u.v.-absorption method. The tissue (about 2 mg.) was placed in a flask containing 10 ml. of 80% (w/w) H_3PO_4 together with ten Teflon balls ($\frac{1}{4}$ in. diam.); the whole was then shaken for 16 hr. at ambient temperature with sufficient vigour to keep the balls in motion but not to cause frothing. Under these conditions the tissue was swollen and dispersed so that unlignified tissue gave a low extinction between 240 and 400 mμ, but lignified tissue gave a lignin spectrum (Fig. 1). Lignin was determined by measuring extinction at 280 mμ, correcting for absorption and scattering of the unlignified tissue, and calculating lignin content on the basis of ϵ 3000 for a unit molecular weight of 180.

Isolation of lignin from artificially lignified parenchyma. (i) An attempt was made to isolate lignin by the Urban (1926) procedure from the eugenol-treated potato parenchyma as follows. Lignified parenchyma (5 g.) was placed in a reagent bottle with 300 ml. of a mixture of conc. HCl (1350 ml.) and 90% (w/w) H_3PO_4 (400 ml.). The bottle was half filled with glass beads and rotated at about 20° for 6 days. The beads were removed by pouring the mixture through a Buchner funnel without a paper, and the lignin was recovered by repeated washing by centrifuging and drying *in vacuo* at 45°.

(ii) Isolation of the lignin by fine grinding in toluene as in the preparation of milled wood lignin according to Björkman (1956) was also attempted. Owing to the small yield obtained, purification was not carried further than the initial precipitation into water from acetic acid solution. The i.r.-absorption spectrum of the eugenol lignin isolated was determined in an NaCl disk by the method of Higgins (1957).

(iii) Methanol-soluble lignin was obtained by heating 50 g. of the lignified parenchyma in 400 ml. of methanol at 150° for 16 hr. The filtered methanol extract was concentrated to 20 ml. and added dropwise to cold (−5°) ether. The precipitated lignin was collected by filtration and dried *in vacuo*.

(iv) A Klason lignin was prepared as in the determination described above from the lignified parenchyma after methanol extraction.

(v) An alkali-soluble lignin was recovered by boiling a specimen of the lignified parenchyma in 0·5 N-NaOH for 1 hr. and acidifying the filtrate. This gave a gelatinous precipitate, which was hydrolysed with 3% (w/v) H_2SO_4 to leave a lignin residue.

(vi) An ethanol lignin was recovered by refluxing 5 g. of the parenchyma in 50 ml. of 3·0% (w/v) HCl in ethanol for 48 hr. After precipitation of the lignin in water, the ether-soluble oils were recovered from the filtrate and examined by the paper-chromatographic method of Kratzl & Schweers (1954).

Nitrobenzene oxidation. Specimens of the lignified parenchyma (0·3 g.) were oxidized with nitrobenzene (0·2 ml.) in 2N-NaOH (7 ml.) for 3 hr. at 160° in stainless-steel bombs. Parallel experiments were done with eugenol, dehydrodieugenol and *Pinus radiata* wood. The vanillin produced was determined by separation on borate-impregnated paper by the method of Bland & Stamp (1955). The insoluble residue left after acidification of the oxidation mixture was extracted with ether, and the ether-soluble matter separated by two-dimensional chromatography on borate-impregnated paper.

Table 1. *Impurities in eugenol before and after purification*

	Coniferaldehyde (%)	Coniferyl alcohol + *iso*eugenol (%)
Eugenol (stock)	0·18	1·0
Purified eugenol	0·004	0·7

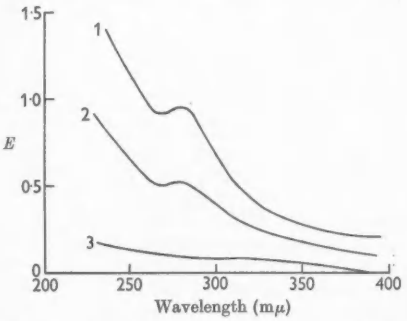

Fig. 1. Ultraviolet-absorption curves of oat-coleoptile tissue in phosphoric acid. 1, Eugenol lignified; 2, eugenol lignified, ethanol-extracted; 3, untreated, ethanol-extracted.

Estimation of p-*hydroxybenzyl alcohol groups.* These were tested for by the *p*-quinonemonochloroimide method of Gierer (1954).

RESULTS

The phloroglucinol–hydrochloric acid colour test was positive for the stock eugenol but negative for the purified material. The results of the analyses given in Table 1 confirm that it contained a significant quantity of coniferaldehyde but that the purification process reduced this to a negligible amount. The artificially lignified tissues stained strongly with phloroglucinol–hydrochloric acid reagent. The u.v.-absorption spectrum of a dispersion of lignified oat-coleoptile tissue in phosphoric acid is shown in Fig. 1.

Determination of lignin by the Klason method on both the oat coleoptiles and the potato parenchyma gave lignin percentages of over 12 % in both cases, but Table 2 shows that the so-called lignins contained nitrogen and that the methoxyl-group percentage was low in each case. The corrected Klason lignin value is based on a methoxyl-group content of 16·0 % for uncontaminated lignin. The lignin values arrived at by u.v. absorption agree better with the corrected lignin figures than with the original yields of Klason lignin for both tissues.

From the corrected Klason lignin value it may be calculated that the potato parenchyma contained artificial lignin corresponding to 39 % of the eugenol added. This shows that the lignin was formed from the eugenol and not only from the coniferaldehyde impurity; however, coniferaldehyde could be an intermediate.

The yield and composition of the isolated lignins are shown in Table 3. They all contained nitrogen.

Table 2. *'Lignin' content of eugenol-treated tissues*

Material	Klason lignin yield (%)	Composition of Klason lignin N (%)	Composition of Klason lignin OMe (%)	Klason lignin yield (% corr. on a basis of 16·0 % of OMe)	Lignin by u.v. absorption (%)
Oat coleoptiles I	14·0	3·88	8·36	7·32	8·5
II	12·0	—	—	—	5·5
Potato parenchyma I	12·7	2·92	12·27	9·71	7·7
II	12·6	1·66	13·79	10·88	—

Table 3. *Yield and composition of isolated potato (eugenol) lignins*

Lignin	Yield as % of parenchyma	C (%)	H (%)	O (%)	N (%)	Total CHON (%)	OMe (%)	OMe* (% corr.)
Urban	15·4	53·54	6·33	34·7	2·22	96·79	7·99	9·28
Klason (after methanol)	8·7	62·04	6·09	25·7	3·84	97·67	11·55	15·20
Ball-mill	0·6	61·66	6·65	28·8	1·64	98·75	11·12	12·39
Alkali	1·1	—	—	—	3·54	—	11·78	15·13
Ethanolysis	0·9	65·47	5·94	25·2	0·86	97·47	13·04	13·78

* Corrected for presence of protein, see text.

The low carbon and methoxyl-group content of the Urban lignin suggest that it is heavily contaminated with carbohydrate material. The yield indicates that it does in fact contain all the lignin but the excessive carbohydrate contamination renders it valueless for chemical investigation. The methanol lignin was obtained in low yield and the ash and nitrogen contents were so high that it also was of no value. The result of the Klason determination after the methanol treatment shows that a large part of the lignin was resistant to the methanol treatment and that it had not been dissolved or degraded to ether-soluble oils.

The ball-mill lignin, recovered by grinding in toluene and extracting with dioxan according to Björkman (1956), shows high carbon, relatively high methoxyl-group content and low ash and nitrogen. These results point to its being a fairly pure lignin but the yield as shown in Table 3 was very low. The i.r.-absorption spectrum of this lignin is shown in Fig. 2 together with those of eugenol and lignin obtained from milled wood of *Pinus radiata*. That of the eugenol lignin clearly resembles that of the *P. radiata* lignin.

Lignin isolated by ethanolysis had the lowest nitrogen content but the yield was also low. Table 3 shows that the total alkoxyl-group content of this lignin, corrected on the assumption that the contaminant is protein containing 16% of nitrogen, does not indicate that any considerable ethylation had taken place, although α-ethoxypropionylguaiacone was present in the ether-soluble oil.

The results of the nitrobenzene oxidation of the artificially lignified potato parenchyma together with those of parallel experiments on *P. radiata* wood, eugenol (I) and dehydrodieugenol (II) are

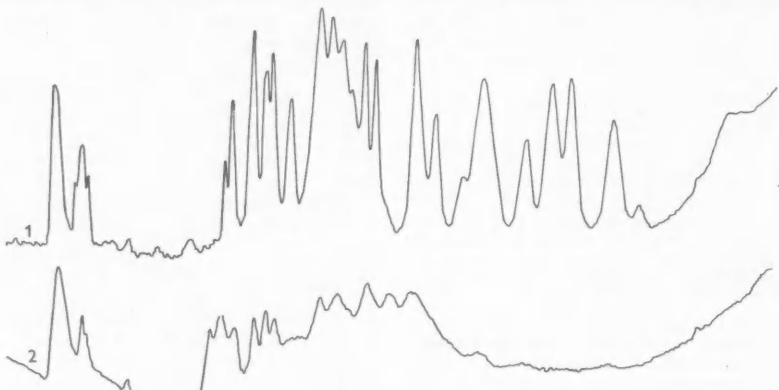

Fig. 2. Infrared-absorption spectra of: 1, eugenol; 2, milled potato-parenchyma eugenol lignin; 3, milled *P. radiata* wood lignin.

Table 4. *Products obtained from artificially lignified potato parenchyma,* Pinus radiata *wood, eugenol and dehydrodieugenol oxidized with nitrobenzene*

	Lignified potato (% of corrected Klason lignin)	*P. radiata* (% of Klason lignin)	Eugenol	Dehydrodieugenol
Vanillin	5·2	19·9	87	0·0
	5·4	20·2	—	—
Dehydrodivanillin	1·1	0·9	0·0	63
	0·7	0·7	—	—

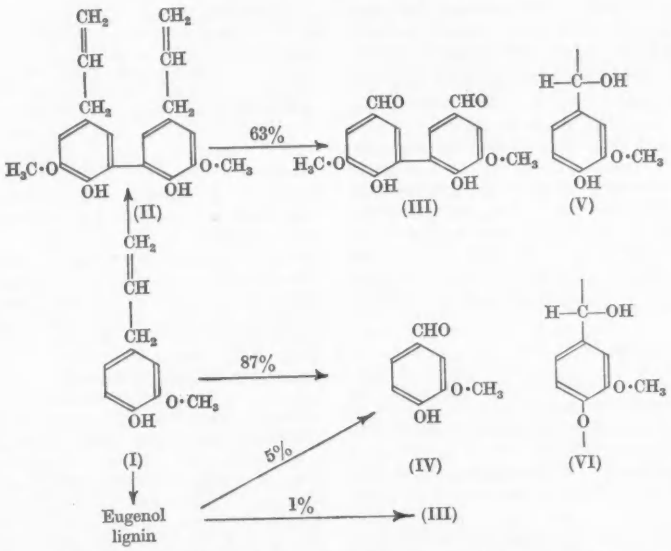

Fig. 3. (I), Eugenol; (II), dehydrodieugenol; (III), dehydrodivanillin; (IV), vanillin; (V), *p*-hydroxybenzyl alcohol type group of gymnosperm lignin; (VI), arylcarbinol group of gymnosperm lignin. Arrows show approximate conversions obtained on nitrobenzene oxidation in the present experiments.

shown in Table 4 and Fig. 3. The yield of vanillin (IV) based on the corrected percentage of eugenol lignin in the potato was about one-fourth of that obtained from *P. radiata* lignin in the same way in both the present and earlier experiments (Bland, 1958). The high yield from eugenol and the zero yield from dehydrodieugenol are in agreement with the results of Pew (1955). The high yield of dehydrodivanillin (III) from dehydrodieugenol also confirms the result of Pew.

The paper-chromatographic examination of ether-soluble material present in the residue obtained after nitrobenzene oxidation disclosed some material (visible in u.v.) at or near the starting line, when the upper phase of *iso*propyl ether–methanol–water (1:1:1, by vol.) was used as solvent. Irrigation in the second direction with methanol–water (1:1, v/v) showed the presence of two materials, one at R_F about 0·7 and one at R_F 0·8. The former corresponded in u.v.-absorption spectrum and R_F with dehydrodivanillin. A sufficient quantity of the compound with higher R_F was isolated by means of thick paper chromatograms to demonstrate that it contained nitrogen and no methoxyl group. This has been identified as *p*-phenylazobenzoic acid and was not derived from the lignin (Bland & Szilagyi, 1960).

The yields of dehydrodivanillin shown in Table 4 suggest that there are about the same number of diphenyl groups in the eugenol lignin as in *P. radiata* lignin.

The artificially lignified potato parenchyma gave no colour reaction with the *p*-quinonemonochloroimide. This indicates that *p*-hydroxybenzyl alcohol groups are absent.

DISCUSSION

The colour reaction with phloroglucinol, the insolubility in 72 % (w/w) sulphuric acid, the infrared and ultraviolet spectra, as well as the ultimate composition of the 'lignin' formed by potato parenchyma from eugenol in the presence of peroxide, all indicate that the eugenol 'lignin' is similar to gymnosperm lignin. On the other hand, its failure to give any colour reaction with *p*-quinonemonochloroimide shows that it contains none of the *p*-hydroxybenzyl alcohol groups (V), which are characteristic of natural gymnosperm lignin (Gierer, 1954). Similarly, the fact that the total alkoxyl-group content of the ethanol lignin did not indicate appreciable ethylation points to the absence of arylcarbinol groups (VI) and their ethers, which are probably the principal groups of natural lignin that can be alkylated (Adler, 1958). The nitrobenzene oxidation experiments of Wacek & Kratzl (1947) and of Kratzl, Hoyes & Silbernagel (1960) with reference compounds show that the low yield of vanillin is in agreement with the

absence of arylcarbinol groups and may be taken to indicate a large number of C–C bonds between the phenylpropane units of the artificial lignin. In view of the readiness with which eugenol forms dehydrodieugenol (II) it might be suspected that a large number of diphenyl links would occur in eugenol lignin, but the low yield of dehydrodivanillin (III) shows that this is not the case. Despite similarities of the eugenol lignin to gymnosperm lignin it does differ from natural lignin in the very important aspects mentioned above and certainly cannot be accepted as a true lignin. This should emphasize the danger of drawing conclusions about natural lignification from experiments in artificial systems.

SUMMARY

1. Artificial lignin has been produced on oat-coleoptile segments and potato parenchyma from purified eugenol in buffered aqueous solution containing hydrogen peroxide.

2. The colour reaction of the eugenol lignin with phloroglucinol, its behaviour in the 72% (w/w) sulphuric acid lignin determination and its ultimate composition were all similar to these characteristics of gymnosperm lignin. Its ultraviolet- and infrared-absorption spectra were similar to those of gymnosperm lignin.

3. Unlike natural lignin, the eugenol lignin has no *p*-hydroxybenzyl alcohol or other arylcarbinol groups that can be ethylated.

4. On oxidation with nitrobenzene the eugenol lignin gave only a low yield of vanillin (5%). The yield of dehydrodivanillin was about the same as from lignin.

5. It is concluded that eugenol lignin is a lignin-like substance, but that it differs from natural lignin in important respects. Conclusions about natural lignin and lignification drawn from experiments with eugenol lignin must therefore be made with the utmost caution.

The author wishes to thank Dr A. B. Wardrop and Mr G. W. Davies for preparation of lignified coleoptiles and the suggestion that larger quantities of eugenol lignin could be produced on potato parenchyma. He is grateful to Mrs M. Szilagyi for technical assistance. The microanalyses were carried out by the Australian Microanalytical Service.

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Biosynthesis of Griseofulvin: the Methylated Benzophenone Intermediates

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Consideration of structural evidence led Birch & Donovan (1953) to suggest that the skeletons of many natural phenolic compounds were derived, at least in part, by the head-to-tail linkage of acetic acid units. Two types of ring closure were distinguished: an aldol type, leading notably to orcinol derivatives, and a *C*-acylation type, leading principally to phloroglucinol derivatives. It was suggested that griseofulvin arose from the head-to-tail linkage of seven acetic acid units and sub-

sequent ring closure of both types, one leading to the phloroglucinol ring and the other to the (potential) orcinol ring. Birch, Massy-Westropp, Rickards & Smith (1958) subsequently observed that [1-^{14}C]acetic acid was incorporated into griseofulvin to the extent of about 1%, with the expected distribution of isotopes.

Barton & Cohen (1957) have proposed a chlorinated benzophenone 5-chloro-6:4′-dihydroxy-2:4:2′-trimethoxy-6′-methylbenzophenone as an intermediate in the biogenesis of griseofulvin. They postulated that one-electron-transfer oxidizing agents would oxidize the benzophenone to the diradical, which would then be coupled to give the intermediate dehydrogriseofulvin and finally griseofulvin by enzymic reduction. Day, Nabney & Scott (1960) later demonstrated this mechanism by chemical synthesis of griseofulvin.

Hockenhull & Faulds (1955) showed that the methoxyl groups of griseofulvin arose by methyl transfer, presumably through the usual biochemical C_1-donor system. Uniform distribution of isotope labelling between the three methoxyl groups suggested that methylation occurred late in the biosynthesis of griseofulvin from a relatively stabilized methyl pool.

Examination by McMaster, Scott & Trippett (1960) of the residual material after extraction of griseofulvin from fermentation broths of mutant strains of *Penicillium patulum* led to the isolation and characterization of a number of new metabolites: dehydrogriseofulvin, griseophenone A, griseoxanthone C, griseophenone Y and dechlorogriseofulvic acid, of which the first two were already known to be intermediates in the chemical synthesis of griseofulvin.

Meanwhile, we had identified the chlorination stage in the biosynthesis of griseofulvin. Extension of this work showed that griseofulvin formation involved a number of benzophenones and that side reactions leading to xanthones could intervene. Biochemical aspects and the structures of these griseofulvin intermediates and by-products are now described. The compounds have been given trivial names to agree with the nomenclature of McMaster *et al.* (1960).

EXPERIMENTAL

Ultraviolet spectra were measured with a Unicam SP. 500 or a Hilger Uvispek spectrophotometer, infrared spectra with either a Perkin–Elmer model 21 or Infracord spectrophotometer. Woelm acid alumina (supplied by L. Light and Co. Ltd.), deactivated to the Brockmann grade quoted in the text, was used. Melting points were determined in tubes and are corrected.

Organisms. Two mutant strains (C4/30, GA766) of *P. patulum* Bainier–Thom C.M.I. 39,809 were used.

Production of inoculum for shake-flask fermentations. The required strain was grown on slopes of Czapek–Dox agar in cylindrical glass bottles (3¼ in. × 1 in.) until sporulation had occurred (7 days at 24°). A small quantity of culture was then inoculated by loop into a 250 ml. conical flask containing 60 ml. of the medium: 2% of brown sugar; 1% of AnalaR $CaCO_3$; 3·8% of corn-steep-liquor solids; pH was adjusted to 5·5 with NaOH and the flask sterilized at 15 lb./in.^{2} for 20 min. After inoculation, the flask was shaken for 48 hr. at 25° on a rotary shaker and 2 ml. of the resulting vegetative mycelium was used to inoculate each flask of fermentation medium.

Standard method of fermentation. This was done in 250 ml. conical flasks containing 40 ml. of the medium: 11% of lactose; 0·3% of KCl; 0·4% of KH_2PO_4; 0·8% of AnalaR $CaCO_3$; corn-steep liquor to give 0·075% of N and distillers' solubles to give 0·075% of N. The flasks were sterilized by autoclaving for 20 min. at 15 lb./in.^{2}. For any given fermentation treatment, pH was determined on the contents of one flask, and this was subsequently adjusted to pH 4·8 with a noted volume of sterile 1% H_2SO_4. This volume of acid was then added to all other flasks of that treatment, thus giving uniform pH for the beginning of all fermentations. There were 3 or 4 replicate flasks of each treatment. Each flask was inoculated as already described and then shaken at 25° on a rotary shaker until the fermentation was completed.

Chromatographic assay. At intervals during the fermentation the contents of the flasks were sampled. Two replicate flasks were sampled at days 5, 7, 9 and 16 or 17, and the remainder at days 9, 12 and 16 or 17. Broth (1 or 2 ml.) was removed into test tubes and extracted by vigorous shaking for 5 min. with an equal volume of chloroform. The tubes were centrifuged and the chloroform layers removed into clean test tubes. The extracts were loaded (0·1 ml. at days 5, 7 and 9; 0·05 ml. at day 12; 0·025 ml. at day 16 or 17) on Whatman no. 1 papers (30 cm. × 50 cm.), which were developed by downward flow. The solvent system, benzene–*cyclo*hexane–methanol–water (5:5:6:4, by vol.) was shaken thoroughly and allowed to separate for at least 2 hr. in a constant-temperature room (70° F). Acetic acid (1 vol.) was then added to 200 vol. of upper layer. The lower layer was used in the bottom of the tanks and the acidified upper layer in the troughs. Immediately before development, a jet of upper-layer solvent was directed just below the origin line on the paper so as to give a broad band of saturation in the path of development. Rates of flow were followed by using a solution (one drop) of α-naphtholbenzein as marker at each side of the papers. Development was usually complete in 6 hr. at 70° F. The papers were then dried and examined under ultraviolet light, first at 3660Å and then at 2537Å.

The benzophenone intermediates appeared as ultraviolet-quenching maroon-coloured spots under radiation of either wavelength. It was observed that the colour saturation of these spots under 3660Å radiation could readily be graded on a scale of 0 to 5 and that these grades of intensity were accompanied by similarly increased areas. This was used as a rough quantitative method of expressing the relative amounts of benzophenone present.

The xanthones and an unidentified substance (*P*) were graded under 2537Å radiation on an arbitrary scale of 0 to 5. The xanthones were assessed by the intensity of their yellow fluorescence and substance *P* by the degree of saturation of its purple ultraviolet-quenching colour.

Spectrophotometric assay. At the end of the fermentation periods (day 16 or 17) the broths were extracted as described below. The contents of each flask, along with 2 drops of octan-1-ol to prevent excessive frothing, were homogenized for approximately 1 min.: 10 ml. of homogenized broth and 80 ml. of acetone were shaken for 30 min. in 250 ml. stoppered flasks containing 10 glass beads (diameter 4–7 mm.); 0·5–1·0 ml. of the supernatant extract was transferred to a tube (6 in. × 1 in.) and evaporated to dryness in a stream of warm air; the residue was dissolved in 1 ml. of *NN*-dimethylformamide and 49 ml. of butyl acetate added; the solution was filtered through Whatman no. 1 paper; the extinction of the filtrate was determined at 288, 292, 296, 298 and 300 mμ. Similar determinations were made on a standard solution of pure griseofulvin in butyl acetate (containing an amount of dimethylformamide such as to make it comparable with the unknown sample). The griseofulvin content of the extracts was determined from the ultraviolet-absorption values by application of the six-point correction procedure of Daly (1961) as an improvement on the method for elimination of irrelevant absorption described by Ashton & Tootill (1956).

Isolation of griseophenone C. A chloride-deficient but otherwise standard fermentation medium with thiourea (30 μg./ml.) added after 96 hr. was used in a 50 gal. vessel inoculated with strain C4/30, and the broth (110 l.; pH 7·6) was filtered on a rotary filter. The wet mycelium was extracted with acetone (2 × 30 l.) and the acetone extract evaporated *in vacuo* to 5 l. (*A*). The broth filtrate was adjusted to pH 3·0 with H_3PO_4 and extracted with chloroform (2 × 30 l.). The extract was evaporated *in vacuo* to 2 l. (*B*).

The acetone solution (*A*) was evaporated further until all the acetone had been removed, and the residual aqueous suspension (about 2 l.) was extracted with chloroform (3 × 1 l.); this chloroform solution and the filtered broth extract (*B*) were combined and evaporated under reduced pressure to about 1 l. A colourless solid (griseofulvin) was separated, and the chloroform solution was extracted with N-NaOH (3 × 500 ml.). The alkaline solution was acidified and extracted with ethyl acetate (3 × 500 ml.). The dried ethyl acetate solution was partly decolorized with activated charcoal and evaporated to about 20 ml. After 24 hr. at room temperature, brown prisms (3·0 g.), m.p. 168–174° (increased to 182–184° by repeated crystallization from ethyl acetate), were separated from the syrupy liquid, which yielded a brown solid (20 g.) on evaporation to dryness. This solid was dissolved in benzene (150 ml.) and filtered through a column of alumina (Grade IV, 150 ml.). Elution with benzene (500 ml.) and benzene–ethyl acetate (2:1, v/v; 500 ml.) yielded a pale-brown solid (9 g.), which was crystallized from ethyl acetate to yield pale-brown prisms (5·5 g.), m.p. 174–178°. Further elution with ethyl acetate yielded a dark-brown solid that could not be purified.

After several recrystallizations from ethyl acetate and finally from benzene, *2:6:4′-trihydroxy-4:2′-dimethoxy-6′-methylbenzophenone* (griseophenone C) was obtained as pale-yellow prisms, m.p. 183–184° [Found: C, 63·1; H, 5·3; OMe, 20·2; mol.wt. (Rast), 276. $C_{16}H_{16}O_6$ requires C, 63·1; H, 5·3; OMe, 20·4%; mol.wt., 304], $\lambda_{max.}$ in ethanol 295 and 228 (infl.) mμ (ε 26 900 and 22 800 respectively), $\lambda_{min.}$ 247 mμ (ε 4360). The infrared spectrum (bromoform) had peaks at 3623, 3448 and 1639 cm.$^{-1}$.

The benzophenone (0·9 g.) was heated under reflux for 2 hr. with acetic anhydride (10 ml.) and pyridine (8 ml.); after dilution with water, a colourless solid was separated. After several recrystallizations from methanol, *griseophenone C triacetate* (0·52 g.) was obtained as colourless prisms, m.p. 155–156° (Found: C, 61·1; H, 5·2; acetyl group 30·8. $C_{22}H_{22}O_9$ requires C, 61·4; H, 5·1; acetyl group, 30·1%). The infrared spectrum (bromoform) had strong peaks at 1756–1750, 1650 and 1215 cm.$^{-1}$.

Methylation of griseophenone C. (i) A solution of griseophenone C (0·8 g.) in dry acetone (60 ml.) was heated under reflux for 24 hr. with anhydrous K_2CO_3 (2·5 g.) and dimethyl sulphate (2 ml.; a further 1 ml. was added after 12 hr.). The reaction mixture was diluted with water and extracted three times with ether. The ethereal solution was extracted with 2N-NaOH (3 × 20 ml.), washed with water, dried and evaporated to yield a yellow oil (0·78 g.). This oil was dissolved in benzene–light petroleum (b.p. 40–60°) (1:1, v/v; 12 ml.) and put on a column of alumina (Grade IV; 20 ml.). Elution with the same solvent (100 ml.) yielded a pale-yellow oil (0·74 g.), that crystallized from methanol to give colourless plates (0·40 g.), m.p. 123–125°. Further recrystallization from methanol yielded *6-hydroxy-2:4:2′:4′-tetramethoxy-6′-methylbenzophenone*, m.p. 124–125° (Found: C, 65·3; H, 6·1; OMe, 37·7. $C_{18}H_{20}O_6$ requires C, 65·1; H, 6·0; OMe, 37·4%), $\lambda_{max.}$ in ethanol 297 and 225 (infl.) mμ (ε 11 900 and 12 000 respectively), $\lambda_{min.}$ 250 mμ (ε 1550). The benzophenone gave a purple colour on treatment with ethanolic $FeCl_3$. The infrared spectrum (bromoform) had strong peaks at 1600 and 1580 cm.$^{-1}$, with a shoulder at 1613 cm.$^{-1}$. The alkaline extract was acidified and extracted with ether. Evaporation of the dried ethereal solution yielded a brown oil (0·08 g.) that could not be purified.

(ii) A solution of diazomethane (about 1 g.) in ether (30 ml.) was added at 0° to a solution of griseophenone C (1·5 g.) in tetrahydrofuran (15 ml.). After 40 hr. at 0°, the excess of diazomethane was decomposed and ether (70 ml.) was added to the ethereal solution before it was extracted with 2N-NaOH. Evaporation of the ethereal solution yielded a yellow oil (1·7 g.), which was chromatographed on alumina as described above. The resultant solid was crystallized from methanol to yield griseophenone C dimethyl ether as colourless prisms, m.p. and mixed m.p. 123–125°. The infrared spectra of the two samples were identical.

Oxidation of griseophenone C. A solution of CrO_3 (3·0 g.) in acetic acid (15 ml.) and water (6 ml.) was slowly added to a solution of griseophenone C (1·0 g.) in acetic acid (15 ml.), heat was evolved, and the temperature of the mixture rose to 60°. After 20 min. at 50°, water (200 ml.) was added to the mixture and the aqueous solution continuously extracted with benzene for 12 hr. Evaporation of the dried benzene solution yielded a brown solid (230 mg.), which was dissolved in benzene–light petroleum (b.p. 40–60°) (2:1, v/v; 5 ml.) and put on a column of alumina (Grade IV; 7 ml.). Elution with the same solvent (25 ml.) yielded a yellow solid (126 mg.), which was crystallized from methanol to yield 3-methoxytoluquinone (57 mg.), m.p. and mixed m.p. with an authentic sample prepared from griseofulvin (Grove, Ismay, MacMillan, Mulholland & Rogers, 1952*a*) 151·5–153·5° (Found: C, 62·8; H, 5·4; OMe, 20·3. Calc. for $C_8H_8O_3$: C, 63·2; H, 5·3; OMe, 20·1%). The infrared spectra of the two samples were identical.

Preparation of griseoxanthone C. (i) A solution of griseophenone C (0·5 g.) in a mixture of ethanol (10 ml.) and water (60 ml.) in a steel bomb was kept at 220° ± 10° for 6 hr.

A pale-yellow solid was separated from the cold mixture and crystallized from ethanol (25 ml.) as yellow needles (0·3 g.; yield 67%), m.p. 255–257°. The crystals were sublimed at 220°±5° and 0·1 mm. Hg, and the sublimate was crystallized from ethanol to yield griseoxanthone C as yellow needles, m.p. 258–259° (Found: C, 66·1; H, 4·5; OMe, 11·4. Calc. for $C_{15}H_{12}O_5$: C, 66·2; H, 4·4; OMe, 11·4%), $\lambda_{max.}$ in ethanol 338 (infl.), 309, 270 (infl.) and 242 mμ (ε 9300, 23 200, 9300 and 37 800 respectively), $\lambda_{min.}$ 280 and 219 mμ (ε 12 000 and 17 400 respectively). The infrared spectrum had major peaks: 3330, 1666, 1626 and 1605 cm.$^{-1}$ (Nujol mull); 3521, 1653, 1618 and 1605 cm.$^{-1}$ (dimethyl sulphoxide); 3226, 1655 and 1605 cm.$^{-1}$ (tetrahydrofuran). McMaster *et al.* (1960) give m.p. 253–255° and $\lambda_{max.}$ 340, 309, 269, 242 mμ (ε 7000, 23 000, 9000 and 37 000 respectively).

(ii) A solution of griseophenone C (1·0 g.) in N-KOH (50 ml.) was heated under reflux and N_2 for 2 hr. The solution was cooled, acidified and extracted with ethyl acetate. Evaporation of the dried ethyl acetate solution yielded a yellow solid (0·85 g.), which was recrystallized from methanol to yield griseoxanthone C (0·76 g.; 85%), m.p. 257·5–259°.

Methylation of griseoxanthone C. A solution of griseoxanthone C (0·59 g.) in tetrahydrofuran was mixed at 0° with a solution of diazomethane (about 250 mg.) in ether (40 ml.). After 2 days at room temperature, the excess of diazomethane was decomposed and the dried ethereal solution evaporated to yield a yellow solid. The solid was recrystallized from methanol to yield lichexanthone (1-hydroxy-3:6-dimethoxy-8-methylxanthone) (0·57 g.; 92%), m.p. and mixed m.p. with a synthetic specimen (see below) 188–189·5° (Found: C, 67·3; H, 5·1; OMe, 21·8. Calc. for $C_{16}H_{14}O_5$: C, 67·1; H, 4·9; OMe, 21·7%). The two specimens had identical infrared spectra (Nujol mull and bromoform).

Synthesis of lichexanthone. Ethyl everninate (6·4 g.), m.p. 71·5–73°, prepared by the methylation of ethyl orsellinate, was hydrolysed with H_2SO_4 for 7 hr. at room temperature (cf. Fischer & Hoesch, 1912) to yield everninic acid (1·1 g.; 20%).

1:3-Dihydroxy-6-methoxy-8-methylxanthone was prepared by the method of Grover, Shah & Shah (1956) from the same quantities of starting materials. A yellow solid (0·19 g.) was obtained and had m.p. 252–258°, increased to 266–269° (decomp.) by several crystallizations from ethyl acetate–light petroleum. Grover *et al.* give m.p. 260–262°. This xanthone had m.p. 227–232° when mixed with griseoxanthone C.

1:3-Dihydroxy-6-methoxy-8-methylxanthone in acetone was methylated with diazomethane in ether (Grover *et al.* 1956) to yield lichexanthone, m.p. 188–189·5° after several crystallizations from ethyl acetate.

Isolation of griseoxanthone C from fermentation broth. The broth (800 ml.) from a chloride-deficient but otherwise standard fermentation medium was extracted with chloroform (3 × 300 ml.) and the chloroform solution evaporated to a small volume (about 40 ml.). After three extractions with 2N-NaOH, the chloroform solution was shown to contain only griseofulvin by paper chromatography and was discarded. The alkaline solution was acidified and extracted with chloroform. Evaporation of the dried chloroform extract yielded a brown solid (0·82 g.) that was dissolved in benzene (6 ml.) and put on a column of alumina (Grade V; 15 ml.). Elution with benzene (110 ml.) and benzene–ether (9:1, v/v; 70 ml.) yielded a brown gum that was shown to be a mixture of griseoxanthone C and griseophenone C by paper chromatography. After several recrystallizations from methanol, griseoxanthone C (5 mg.) was obtained as yellow needles, m.p. 257·5–259°, undepressed on admixture with an authentic specimen. The two specimens had identical infrared spectra (Nujol mull) and the same R_F when run on the same paper chromatograms.

Extraction of griseophenones A and B. A standard shake-flask fermentation medium inoculated with strain C4/30 was harvested on the seventh day, when the chromatograms showed good yields of griseophenones A and B with minimum amount of griseophenone C. The mycelium was separated from the broth (5 l.), to which kieselguhr had been added to aid the separation, and the mycelium–kieselguhr was then extracted with acetone (2 × 2 l.). The acetone was removed by evaporation under reduced pressure and the residual aqueous suspension extracted with chloroform (3 × 400 ml.). The broth filtrate (4 l.) was also extracted with chloroform (3 × 1·2 l.), and the two chloroform solutions were combined and concentrated to a small volume (about 400 ml.). The chloroform solution was then extracted with 2N-NaOH (3 × 100 ml.); 2N-HCl (200 ml.) was slowly added to the cooled alkaline solution, which was then saturated with CO_2. The resultant solution (pH 7·5) was extracted with chloroform (3 × 200 ml.), and the dried chloroform extract evaporated to yield a brown gum (4·9 g.). The gum was leached several times with boiling benzene (total 120 ml.), and the cooled solution filtered through alumina (Grade IV; 30 ml.). Elution with benzene (300 ml.) and benzene–ethyl acetate (4:1, v/v; 200 ml.) yielded a brown solid (3·9 g.).

Countercurrent separation of griseophenones A and B. The distribution was carried out in all-glass 50-tube apparatus (40 ml. each phase) with a benzene–methanol–water–N-HCl (200:200:100:1, by vol.) solvent system. The solid (3·9 g.), previously purified on alumina, was dissolved in upper phase (160 ml.) and put into tubes 1–4 of the apparatus, which had been filled with lower phase. The last tube of the apparatus was coupled to a fraction collector actuated when the machine was in the 'decant' position. After 210 transfers, the ultraviolet absorption of the upper-phase fractions at 295 mμ was measured and plotted to give the distribution curve shown in Fig. 1. Fractions 10–54 were combined and evaporated to yield a brown solid (1·58 g.), shown by paper chromatography to be griseophenone A. The solid was crystallized from benzene to yield 5-chloro-6:4′-dihydroxy-2:4:2′-trimethoxy-6′-methylbenzophenone (0·80 g.), m.p. 213–214° undepressed on admixture with an authentic specimen (Found: C, 57·7; H, 4·7; OMe, 26·4; Cl, 10·0. Calc. for $C_{17}H_{17}O_6Cl$: C, 57·9; H, 4·8; OMe, 26·4; Cl, 10·1%). McMaster *et al.* (1960) give m.p. 212–214° for griseophenone A. The two specimens had identical infrared spectra (tetrahydrofuran) and the same R_F when run on the same paper chromatogram.

Evaporation of fractions 55–120 yielded a yellow solid (1·22 g.) that was shown by paper chromatography to be griseophenone B. The solid was crystallized from benzene to yield *5-chloro-2:6:4′-trihydroxy-4:2′-dimethoxy-6′-methylbenzophenone* (0·85 g.) as yellow prisms, m.p. 204·5–205·5° (Found: C, 56·9; H, 4·4; OMe, 18·3; Cl, 10·3. $C_{16}H_{15}O_6Cl$ requires C, 56·7; H, 4·4; OMe, 18·3; Cl, 10·5%), $\lambda_{max.}$ in ethanol 293 and 235 (infl.) mμ (ε 21 800 and 19 300 respectively), $\lambda_{min.}$ 255 mμ (ε 4900). The infrared spectrum had peaks at 3448, 1626 (shoulder), 1610 and 1575 cm.$^{-1}$

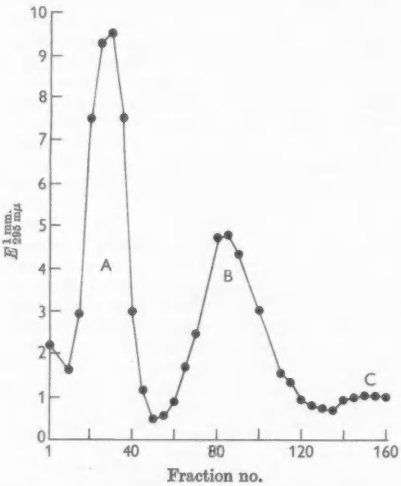


Fig. 1. The separation of griseophenones A, B and C by countercurrent distribution. A, B and C refer to the corresponding griseophenones.

(Nujol mull) and 3584, 3425, 1618 (shoulder) and 1608 cm.$^{-1}$ (tetrahydrofuran).

Fractions 121–129 were evaporated to yield a brown solid (64 mg.) that was found to be a mixture of griseophenones B and C on paper chromatography. Fractions 130–160 and the contents of tubes 26–50 were evaporated to yield a dark-brown solid (442 mg.), from which griseophenone C (146 mg.), m.p. 179–182°, was obtained on crystallization from benzene.

Extraction of griseophenones A, B and C and griseoxanthone C from large-scale fermentation residues. The residues were a dark-brown thick gum obtained by evaporating to dryness the mother liquors from a large-scale crystallization of crude griseofulvin.

The residues (about 250 g.) were dissolved in chloroform (1·3 l.), filtered to remove some resinous material and extracted with 0·5N-NaOH (total 2·5 l.). The chloroform solution was concentrated and, in the cold, impure griseofulvin separated. This process was repeated until the volume of the solution was about 100 ml. and crude griseofulvin (about 75 g.) was obtained. The chloroform solution was extracted with 2N-HCl (3 × 40 ml.), and the acid solution made alkaline with NaOH and then extracted with chloroform (3 × 50 ml.). Evaporation of the chloroform solution yielded a brown solid (5·1 g.) that was shown to contain a large amount of substance *P* by paper chromatography. The alkaline solution was adjusted to pH 7·5 with H_3PO_4 and extracted with chloroform (3 × 800 ml.). Evaporation of this chloroform solution yielded a dark-brown gum (31·5 g.) shown to contain griseophenones A, B, C and griseoxanthone C by paper chromatography.

The gum was dissolved in benzene (150 ml.) and filtered through alumina (Grade IV; 300 ml.). Elution with benzene (800 ml.) and benzene–ethyl acetate (4:1, v/v; 600 ml.) yielded a brown solid (19·0 g.) that was washed three times with boiling benzene (total 60 ml.). The pale-brown solid that separated from the cold benzene extract and the solid left after the benzene washings were combined and separated by countercurrent distribution. The dark-coloured benzene solution was discarded.

Countercurrent distribution of extracts from large-scale fermentation residues. The distribution was carried out in five separating funnels by the 'diamond' pattern of Bush & Densen (1948) with a benzene–methanol–water–2N-HCl system (500:600:500:1, by vol.). The volumes of the upper phase (180 ml.) and the lower phase (270 ml.) for each equilibration were chosen so that the equation $\sqrt{(K_A r \times K_C r)} = 1$ [where K_A = partition coefficient of griseophenone A = 3·8 and K_C = partition coefficient of griseophenone C = 0·6 so that r = (volume of upper phase)/(volume of lower phase) = 2/3] was satisfied.

Some of the brown solid (8·3 g.) was dissolved in the upper phase (180 ml.) and was separated into ten fractions when the distribution was complete. The contents of each fraction were examined by paper chromatography and fractions of similar composition combined. Fractions 1–4 (1·70 g.) were crystallized from benzene to yield griseophenone C (1·17 g.), m.p. 173–176° increased to 181–183° by further crystallization from ethyl acetate–benzene. Fractions 7–10 (5·82 g.) were also crystallized from benzene to yield griseophenone A (2·06 g.), m.p. 210–212°. Paper chromatography of these benzene mother liquors showed the presence of griseoxanthone C and griseophenone A. The mother liquors were evaporated to leave a brown solid (3·7 g.). This solid and the remainder of the benzene-washed solid were subjected to another 'diamond' countercurrent distribution, with ten separating funnels. The solvent system was benzene–methanol–water–2N-HCl (600:600:300:1, by vol.), for which K_A is 1·78 and K_C is 0·34 so that r is 1·29, calculated from the above expression. The solid (9·5 g.) was dissolved in upper phase (256 ml.) and equilibrated with lower phase (200 ml.). Twenty fractions were obtained and treated as already described to yield: (*a*) Fractions 2–9, griseophenone C (819 mg.), m.p. 176–178° increased to 182–183° by further crystallization. (*b*) Fractions 10–19, griseophenone A (1·52 g.), m.p. 210–212°. The mother liquors were concentrated and a yellow solid (1·92 g.), m.p. 181–198°, was obtained. This solid was crystallized several times from methanol to yield griseoxanthone C (206 mg.), m.p. 256–258°.

Fractions 5 and 6 (719 mg.) from the first 'diamond' countercurrent distribution were shown to be a mixture of griseophenones A, B and C by paper chromatography and were fractionated by countercurrent distribution under the conditions described for the isolation of griseophenones A and B (see above). The crude, combined fractions were crystallized from benzene to yield griseophenones A (180 mg.), m.p. 212–213°, B (100 mg.), m.p. 188–190°, and C (170 mg.), m.p. 179–182°.

RESULTS

Observations on shake-flask fermentations. Chloride deficiency, induced by omission of potassium chloride from the standard fermentation medium, affected the metabolism of the two strains C 4/30 and GA 766. Biosynthesis of griseofulvin (R_F 0·85) was markedly inhibited in both, the final mean yields

being diminished by 78–81 %. The formation of a small amount of griseofulvin in the absence of added chloride was due to the presence of some chloride in the corn-steep liquor and distillers' solubles used as medium ingredients. The diminution in griseofulvin biosynthesis was accompanied by altered proportions of griseophenones, griseoxanthones and substance *P*.

Strain C4/30, in the standard fermentation medium, produced griseophenone C (R_F 0·25), griseophenone B (R_F 0·45) and griseophenone A (R_F 0·60), together with a substantial quantity of *P* (R_F 0·10). The effect of chloride deficiency could be seen by the fifth day and resulted in a marked accumulation of griseophenone C, but an absence of griseophenone B and a substantial diminution in griseophenone A. By the twelfth day there was also substantial diminution in substance *P*. These changes in the chloride-deficient fermentation broth were associated with the advent of griseoxanthone C (R_F 0·75) at the ninth day and its gradual accumulation up to the seventeenth day.

Strain GA766 behaved essentially in the same way except that larger amounts of griseophenone C, griseophenone B and substance *P* were formed in the standard fermentation broth. Enhanced synthesis of griseophenone C by this strain masked the effect of chloride deficiency on griseophenone C formation at the seventeenth day, but accumulation of this material between the fifth and twelfth days was obvious. This strain was also notable for the synthesis of a new substance, 'griseoxanthone B' (R_F 0·80), in the standard fermentation broth by the seventeenth day, but formation of this substance was prevented by chloride deficiency.

Subsequently it was shown that these effects of chloride deficiency could be simulated by addition of thiouracil or copper-chelating substances, such as thiourea, sodium diethyldithiocarbamate, picrolonic acid, rubeianic acid and salicylaldoxime, or by reduced aeration.

It was more difficult to determine the effects of chloride deficiency with the parent strain, *Penicillium patulum* Bainier–Thom CMI 39,809, as it had a much lower potential for griseofulvin production. Nevertheless, a considerable quantity of griseophenone C was observed at the seventh day in chloride-deficient fermentation broths.

The accumulation of griseophenone C when griseofulvin biosynthesis was inhibited by deficiency of chloride suggested that this benzophenone might be the substrate for the chlorination reaction. If this were so, then addition of chloride at various times to a chloride-deficient fermentation broth would be expected to permit identification of further griseofulvin intermediates by observation of the sequence to removal of the metabolic block.

An experiment was performed with strain C4/30 when 0·3 % of potassium chloride was added at either the fifth, seventh or ninth day to a chloride-deficient standard fermentation broth. The fermentation medium without added potassium chloride showed by the fifth day the typical chloride-deficient metabolic picture already described. Addition of potassium chloride to the chloride-deficient fermentation broth at the fifth day resulted within 48 hr. in a diminution of griseophenone C accompanied by the presence of griseophenone B in large quantity and a small increase in griseophenone A. This diminution in griseophenone C was associated with absence of griseoxanthone C, as opposed to its appearance in the chloride-deficient fermentation broth at the seventh day and its gradual accumulation thereafter. Delay in the addition of potassium chloride to the chloride-deficient fermentation broth until the seventh or ninth day gave essentially similar results as addition at the fifth day, but resumption of the chlorination reaction suffered a lag period that increased progressively with the span of chloride deficiency.

The rate at which the chlorination of griseophenone C progressed was reflected in the mean yields of griseofulvin on the sixteenth day. The chloride-deficient medium afforded only 22 % of the mean amount of griseofulvin in the standard fermentation medium but addition of potassium chloride to the chloride-deficient fermentation broth at the fifth, seventh or ninth day increased the relative mean yields to 66, 29 and 26 % respectively.

Substitution of potassium iodide, bromide or fluoride at various concentrations in place of potassium chloride additions to similar chloride-deficient fermentation media did not yield any detectable quantity of iodo-, bromo- or fluoro-griseofulvin, although it seemed that iodide, and more particularly bromide, took part to a small extent in the halogenation reaction, since they led to the appearance of traces of ultraviolet-quenching substances having R_F values similar to that of griseophenone B. It may therefore be concluded that in this strain the halogenation of griseophenone C is specific for chlorine, but that iodine, and to a greater extent bromine, can participate to a minor degree.

Since bromide and iodide had replaced chloride in the chloride-deficient fermentation medium to a small extent only, it seemed possible that these halogens might in fact antagonize the chlorination reaction in the griseofulvin fermentation.

The addition of either 0·21 % of potassium bromide or 0·18 % of potassium iodide to the standard fermentation broth already provided with abundant chloride resulted in accumulation of griseophenone C and concomitant diminution of substance *P* and

griseophenones B and A, thus presenting a picture similar to that already observed in chloride-deficient conditions. Similarly, the metabolic block caused by bromide or iodide resulted in markedly decreased biosynthesis of griseofulvin, the yields being only 36 and 52 % respectively of that in the standard fermentation medium, accompanied by griseoxanthone C formation in the later stages of fermentation.

None of the experiments in which the chlorination reaction was inhibited provided any evidence for a major biosynthetic route from griseophenone C to dechlorogriseofulvin. This was subsequently confirmed by growth of strain C4/30 in a chloride-deficient fermentation medium prepared from substantially chloride-free corn-steep liquor (British Patent 773,453), although in this instance small amounts of dechlorogriseofulvin were observed by chromatography of broth extracts. This substance had slightly lower R_F and darker-blue ultraviolet fluorescence than griseofulvin and the crude solid gave a blue–violet colour when treated with concentrated nitric acid (MacMillan, 1953).

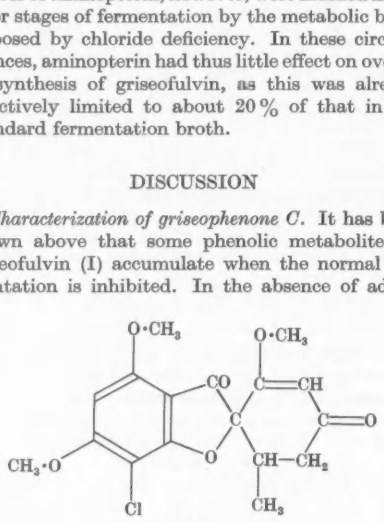
Further information was obtained by identifying the methylation stages in griseofulvin biosynthesis. With strain C4/30, addition of 0·1 % of choline chloride to the standard fermentation medium at 0, 48 or 96 hr. resulted in markedly faster biosynthesis of griseophenone C. The increased rate of formation of griseophenone C on addition of a methyl donor suggested that a folic acid antagonist, such as aminopterin, might have an inhibitory action on methylations in the griseofulvin fermentation. The standard fermentation medium, however, usually produced substantial quantities of griseophenone A, which might mask the inhibitory effect of aminopterin if this were exerted at some point in benzophenone synthesis.

Under conditions of optimum chloride concentration but with lactose reduced to 7 %, aminopterin (2, 4 or 8 μg./ml. added at 0 hr.) diminished biosynthesis of griseofulvin, the mean yields falling to between 64 and 73 % of that from the standard fermentation medium, and led to accumulation of griseophenones B and C between the twelfth and eighteenth days. Aminopterin, however, also depressed the overall biosynthesis of griseophenones and substance *P* during the earlier stages of fermentation. Nevertheless, the ratio of griseophenone A to griseophenone B was substantially less throughout the aminopterin treatment.

The results obtained with the same additions of aminopterin to a chloride-deficient medium were similar to those obtained in a chloride-deficient medium without aminopterin: thus griseophenone C accumulated together with griseoxanthone C. These changes were accompanied by diminutions in biosynthesis of griseophenone A and substance *P* and by absence of griseophenone B, as expected. Aminopterin, however, superimposed its inhibitory action on these effects of chloride deficiency and caused accumulation of griseophenones A and B, but now accompanied by diminution in griseophenone C and griseoxanthone C production during the earlier stages of fermentation. The inhibitory effects of aminopterin, however, were masked in the later stages of fermentation by the metabolic block imposed by chloride deficiency. In these circumstances, aminopterin had thus little effect on overall biosynthesis of griseofulvin, as this was already effectively limited to about 20 % of that in the standard fermentation broth.

DISCUSSION

Characterization of griseophenone C. It has been shown above that some phenolic metabolites of griseofulvin (I) accumulate when the normal fermentation is inhibited. In the absence of added



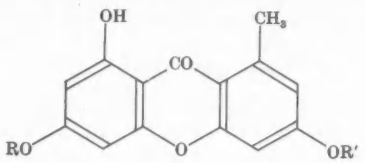
(I)

chloride, griseophenone C accumulated to the exclusion of the other metabolites. This substance contained two methoxyl groups. The infrared spectrum of its triacetate had peaks (1756–1750 and 1215 cm.$^{-1}$) consistent with the presence of phenolic acetate groupings and another peak (1650 cm.$^{-1}$) consistent with the carbonyl frequency of an aryl or diaryl ketone with several methoxyl substituents. A similar peak (1613 cm.$^{-1}$) in the dimethyl ether may be attributed to a carbonyl group hydrogen bonded with an *ortho* hydroxyl group, which is also consistent with its insolubility in alkali. Similar hydrogen bonding must occur in the parent substance to give the lowered frequency (1639 cm.$^{-1}$) of the carbonyl peak. The ultraviolet spectrum of the metabolite is similar to that of methyl phloracetophenone recorded by Morton & Sawires (1940) and, in part, to that of griseofulvin (Grove, MacMillan, Mulholland & Rogers, 1952*b*). It was probable that griseophenone C was a trihydroxydimethoxymethylbenzophenone.

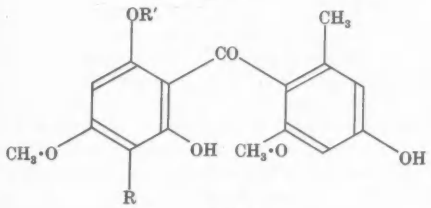
On oxidative degradation, similar to that used on griseofulvin by Grove *et al.* (1952*a*), the benzophenone was readily oxidized, and under mild conditions chromic oxide oxidation yielded 3-methoxy-

toluquinone. The isolation of this toluquinone proves that one aromatic ring of the benzophenone is substituted with one hydroxyl, one methoxyl and one methyl group, and, by analogy with griseofulvin, the other aromatic ring is probably a phloroglucinol residue of which one hydroxyl group has been methylated.

Some benzophenones may be degraded to substituted benzoic acids when fused with potassium hydroxide, but fusion of this benzophenone yielded a small quantity of a non-acidic yellow crystalline solid. This yellow solid was also obtained when the benzophenone was heated with potassium hydroxide solution under various conditions or with water at 220° in a closed tube. An almost quantitative yield was obtained when the benzophenone was heated under reflux with N-potassium hydroxide for 2 hr. Methanol had been eliminated and the ultraviolet spectrum of the substance was similar to those reported by Yates & Stout (1958) for 1-hydroxyxanthones. A monomethyl ether of the substance was identical with a specimen of lichexanthone (II; R = R′ = Me) synthesized by the method of Grover *et al.* (1956).



(II)



(III)

Only two structures are possible for xanthones whose monomethyl derivatives are lichexanthone. One of these, 1:3-dihydroxy-6-methoxy-8-methylxanthone (II; R = H; R′ = Me), was an intermediate in the synthesis of lichexanthone and was different from the substance derived from the metabolite. Thus it follows that this substance, griseoxanthone C, must have the structure 1:6-dihydroxy-3-methoxy-8-methylxanthone (II; R = Me; R′ = H). [McMaster *et al.* (1960) isolated griseoxanthone C from the residue obtained by evaporation of mother liquors from the crystallization of crude griseofulvin. The work described above was carried out concurrently with theirs, but independently of it.] The position of the methoxyl group in this xanthone fixes the position of the methoxyl group in the phloroglucinol residue of the benzophenone. The methoxyl group already proved to be present in the other ring of the benzophenone was therefore eliminated in the formation of griseoxanthone C and must have been *ortho* to the carbonyl group. From the structure of griseoxanthone C, the methyl group must also be *ortho* to the carbonyl group, so that the structure of griseophenone C is 2:6:4′-trihydroxy-4:2′-dimethoxy-6′-methylbenzophenone (III; R = R′ = H).

The elimination of methanol from a benzophenone to yield a xanthone has been reported by Barton & Scott (1958), who isolated a xanthone carboxylic acid from dihydroerdin after treatment with boiling N-sodium hydroxide, and by Hassall & McMorris (1959), who obtained a dichloroxanthone when dihydrogeodoxin was kept at 125° for 5 min. with sulphuric acid (80%). Nishikawa (1937) had isolated a substance that was probably a xanthone carboxylic acid from the potassium hydroxide fusion of sulochrin, a benzophenone from the mycelium of *Oospora sulphurea-ochracea*. In these examples, the elimination of methanol has been facilitated by an acid or base, but we know of no previous reports of the elimination of methanol in a sealed tube at high temperature. This method was first described by Dutta & Watson (1911) for the preparation of xanthones by elimination of water from *oo′*-dihydroxybenzophenones, and Grover, Shah & Shah (1955) have reported that the conversion is almost quantitative.

Characterization of griseophenone B. Griseophenone B had a molecular formula $C_{16}H_{15}O_6Cl$, identical with that of griseophenone C when chlorine is substituted for one hydrogen. Since it has also been shown that griseophenone B is formed from griseophenone C during the fermentation and is subsequently converted into griseophenone A, it follows that griseophenone B is 5-chloro-2:6:4′-trihydroxy-4:2′-dimethoxy-6′-methylbenzophenone (III; R = Cl; R′ = H).

Biosynthesis of griseofulvin. It may be concluded from the above that biosynthesis of griseofulvin proceeds from griseophenone C through griseophenones B and A to griseofulvin.

Chlorination has been shown to be a key reaction in this biosynthesis. The marked accumulation of griseophenone C accompanied by absence of griseophenone B and a marked fall in griseofulvin yield under chloride-deficient conditions strongly suggests that griseophenone C is the substrate for the chlorination reaction. This is supported by the observation that chloride ions supplied to a chloride-deficient fermentation medium that has accumu-

lated griseophenone C at the expense of griseofulvin formation can restart griseofulvin biosynthesis by progression first through griseophenone B and then through griseophenone A. Further support for this metabolic sequence is found in the fact that accumulation of griseophenone B in the presence of aminopterin is accompanied by increase in griseophenone C and a corresponding depression in the relative amount of griseophenone A formed.

The chlorination reaction in which griseophenone C is converted into griseophenone B appears to be similar to the chlorination stage in the fermentation of chlorotetracycline: both reactions are inhibited by bromide or iodide ions, thiouracil, reduced aeration or copper-chelating substances (Di Marco & Pennella, 1959), such as thiourea, sodium diethyldithiocarbamate, picrolonic acid, rubeianic acid and salicylaldoxime.

The two methoxyl groups of griseophenone C appear to be formed through the usual biochemical C_1-donor system, because formation of this intermediate is accelerated by choline chloride but inhibited by aminopterin, and the latter might be expected to block methylation owing to antagonism of folic acid. Similarly, the same C_1-donor system appears to be involved in the methylation reaction whereby griseophenone B is converted into griseophenone A, since this also is inhibited by aminopterin.

The aminopterin-induced metabolic block between griseophenone A and griseofulvin is unlikely to be due to interference with a methylation process, since this benzophenone already contains all the methoxyl groups of griseofulvin. It is possible that biosynthesis of griseofulvin from griseophenone A may be an oxidation–reduction reaction dependent on folic acid.

There are several side reactions that can intervene in the biosynthesis of griseofulvin. Thus whenever griseophenone C reaches a high concentration, regardless of the method of inhibition employed, formation of griseoxanthone C soon follows. Similarly, it was observed that strain GA766 formed 'griseoxanthone B', but its formation was prevented under chloride-deficient conditions.

Griseoxanthone C has been shown to be readily formed *in vitro* from griseophenone C by elimination of methanol. Similarly, 'griseoxanthone B' has been shown to be formed readily from griseophenone B *in vitro*, but we have, as yet, only recognized this substance by paper chromatography. It has an orange–yellow fluorescence under ultraviolet light and R_F 0·80, compared with the golden-yellow fluorescence and R_F 0·75 of griseoxanthone C. It seems likely that 'griseoxanthone B' may contain chlorine, since it is formed *in vitro* from griseophenone B and its biosynthesis is inhibited by the chloride-deficient conditions that caused accumulation of griseoxanthone C. It is therefore possible that griseoxanthones C and B formed during our fermentations may be due to elimination of methanol from griseophenone C and griseophenone B respectively, in side reactions initiated when the subsequent reactions leading to griseofulvin are rate-limiting.

Another possibility is that the griseoxanthones may be formed *in vivo* by removal of water. Thus a benzophenone precursor of griseophenone C might lose water to give an unmethylated xanthone, which in turn could give griseoxanthone C by methylation or 'griseoxanthone B' by chlorination and methylation.

None of the experiments in which the chlorination reaction was inhibited provided any evidence for a major biosynthetic route from griseophenone C to dechlorogriseofulvin, although a small amount of this compound was observed when substantially chloride-free corn-steep liquor was used in the chloride-deficient fermentation medium. This side reaction might vary quantitatively with the strain of organism used and could account for the dechlorogriseofulvin isolated from surface cultures by MacMillan (1953). Subsequent hydrolysis of dechlorogriseofulvin could afford the dechlorogriseofulvic acid found by McMaster *et al.* (1960).

SUMMARY

1. Inhibition by appropriate methods of griseofulvin shake-flask fermentations and paper chromatography of the broth extracts showed that decreased yield of griseofulvin was accompanied by increased amounts of other metabolites.

2. Chlorination was shown to be a key reaction in griseofulvin biosynthesis. Inhibition of this reaction resulted in accumulation of griseophenone C, 2:6:4′-trihydroxy-4:2′-dimethoxy-6′-methylbenzophenone.

3. Chlorination of griseophenone C in the course of the fermentative production of griseofulvin afforded griseophenone B, which was shown to be 5-chloro-2:6:4′-trihydroxy-4:2′-dimethoxy-6′-methylbenzophenone.

4. Griseophenone B was shown to be methylated in the fermentation to give griseophenone A, which was found to be 5-chloro-6:4′-dihydroxy-2:4:2′-trimethoxy-6′-methylbenzophenone.

5. Griseophenone A was shown to be an intermediate in griseofulvin biosynthesis, and its conversion into griseofulvin was inhibited by aminopterin.

6. A side reaction in which griseoxanthone C (1:6-dihydroxy-3-methoxy-8-methylxanthone) was formed, intervened whenever griseophenone C reached a high concentration. Griseoxanthone C was isolated after a fermentation in which the chlorination of griseophenone C was inhibited. The

same compound was also prepared in good yield by various methods from griseophenone C, and its monomethyl ether was identical with lichexanthone.

7. The sequence established for the biosynthesis of griseofulvin is: griseophenone C → B → A → griseofulvin.

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Studies of Fatty Acid Oxidation

7. THE EFFECTS OF FATTY ACIDS ON THE PHOSPHATE METABOLISM OF SLICE AND MITOCHONDRIAL PREPARATIONS OF RAT LIVER*

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The mechanism of fatty acid oxidation in mammalian tissues has been the subject of detailed investigation in recent years (see, for example, the review by Lynen, 1955), and it has been shown that they must undergo an initial activation step involving the participation of adenosine triphosphate to form acyl-coenzyme A derivatives. It was reasonable therefore to infer that inhibition of regeneration of adenosine triphosphate would lead to inhibition of fatty acid oxidation such as has been shown to occur in the presence of agents which uncouple oxidation from phosphorylation (Cross, Taggart, Covo & Green, 1949; Lehninger, 1951). Quastel & Wheatley (1933) observed that fatty acids when present at concentrations above an optimum value gave rise to an inhibition of respiration of guinea-pig-liver slices. Lehninger (1945, 1951) made a similar observation on the oxidation of fatty acids by liver particles, and suggested that the fatty acids might act as uncoupling agents. It was later shown that decanoate decreases the phosphate/oxygen ratio for pyruvate oxidation in rat-brain and -kidney mitochondria (Scholefield, 1956) and that fatty acids stimulate the latent adenosine-triphosphatase activity of liver mitochondria (Pressman & Lardy, 1956). Further work (Creaser & Scholefield, 1960) showed that decanoate strongly inhibits phosphate incorporation into all

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the organic phosphate fractions of Ehrlich ascites-carcinoma cells. It was decided to extend these studies on the inhibitory effects of fatty acids to determine whether the effects of several short- and medium-chain-length fatty acids on the respiratory activity of the cell could be correlated with their effects on specific aspects of phosphate metabolism.

MATERIALS AND METHODS

Animals. Adult hooded rats of a local inbred strain, weighing approx. 200 g., were used throughout this investigation.

Chemicals. All common chemicals were of reagent grade and were used without further purification. ATP and ascorbic acid were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., cytochrome *c* from Wyeth Inc., Philadelphia, Pa., U.S.A. and hexokinase from the Sigma Biochemical Co., St Louis, Mo., U.S.A. Radioactive phosphate was obtained from Charles E. Frosst and Co., Montreal, P.Q., Canada. It was boiled with N-HCl for 1 hr. in a water bath to decompose pyrophosphate and neutralized before use.

All fatty acids were used as the potassium salts and were prepared and purified as described by Scholefield (1956). [1-^{14}C]Decanoate was purchased from Merck and Co. Ltd., Montreal. It was neutralized with $NaHCO_3$ and sufficient carrier potassium decanoate was added to give a final concentration of 20 mM. The radioactivity of this solution was approx. 4·2 μc/ml. (0·21 μc/μmole). All substrates and reagents were adjusted to pH 7·4 before use.

Tissue preparations. Rats were stunned and immediately killed by decapitation. Liver or kidneys were quickly removed from the animal and chilled in cracked ice. Tissue slices were cut by means of a Stadie–Riggs tissue slicer (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) and immediately placed on top of a dry Petri dish containing cracked ice. The slices were then weighed on a torsion balance and placed directly in chilled Warburg vessels containing the experimental media.

Rat-liver mitochondria were isolated according to the method of Hogeboom, Schneider & Pallade (1948) in 0·25 M-sucrose.

Oxygen uptake was measured with the conventional Warburg apparatus at 37° in an atmosphere of O_2 (slices) or air (mitochondria). Experiments were repeated at least three times and mean values are quoted. The individual values did not differ from the mean by more than 10%.

Calcium was omitted from the Krebs–Ringer incubation medium, the final concentrations of the various salts being: NaCl, 145 mM; KCl, 5·8 mM; KH_2PO_4, 1·5 mM; $MgSO_4$, 1·5 mM. Sodium phosphate buffer, pH 7·4, was added to give a final concentration of 10 mM and final pH 7·3. The medium for measuring oxidative phosphorylation in mitochondria was similar to the one described by Judah & Rees (1953). In a final volume of 3 ml. it contained: $MgSO_4$, 6·7 mM; KCl, 75·0 mM; ATP, 1·67 mM; cytochrome *c*, 3·5 μM and sodium phosphate buffer, pH 7·4, 10 mM. Glucose (20 mM) and hexokinase (1 mg.) were used as the phosphate-acceptor system. Other substrates were added as indicated in the text. Inorganic phosphate was estimated by the method of Fiske & Subbarow (1929). Cytochrome *c*-oxidase activity, the associated oxidative phosphorylation and the phosphorylation occurring during the oxidation of succinate, were measured by the methods described by Lehninger, ul Hassan & Sudduth (1954), Borgstrom, Sudduth & Lehninger (1955) and Hunter & Ford (1955).

Calculation of results. Most of the results are expressed in terms of μm-moles of phosphate incorporated into the various fractions/100 mg. wet wt. of tissue, calculated from the formula

$$\mu\text{m-moles incorporated/100 mg. wet wt. of tissue} = \frac{\text{counts/min./fraction}}{\text{counts/min./}\mu\text{mole of phosphate in the incubation medium}} \times \frac{100}{\text{wet wt. of slice (mg.)}}.$$

All experiments were performed at least twice and either typical or mean values are quoted.

Study of incorporation of ^{32}P into slices. The slices were incubated as described above. ^{32}P (50–100 μc) was tipped into the experimental medium in Warburg vessels after an initial equilibration period of 10 min. At the end of the incubation, the slices were quickly removed and washed twice with 5 ml. of cold Krebs–Ringer solution. The washed slices were homogenized with 5 ml. of 5% cold trichloroacetic acid, the acid-insoluble fraction was isolated, the acid-soluble phosphate compounds were separated by paper chromatography and the radioactivity of each was measured with a thin end-window Geiger–Müller tube and Tracerlab 123 scaler as previously described (Creaser, de Leon & Scholefield, 1959), to an accuracy of within 5%.

Metabolism of [1-^{14}C]decanoate. The [1-^{14}C]decanoate was placed in the main compartments of Warburg flasks along with the other constituents of the incubation medium. The side arm contained 0·3 ml. of 2 N-H_2SO_4 and 0·5 ml. of 50%

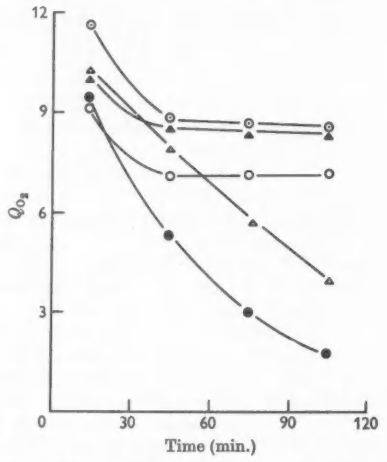

Fig. 1. Effects of potassium decanoate on the respiratory activity of rat-liver slices. ○, No decanoate added; ⊙, 0·66 mM-decanoate; ▲, 1·33 mM-decanoate; △, 2·67 mM-decanoate; ●, 4 mM-decanoate. Q_{O_2} values were calculated for the 30 min. intervals indicated.

(w/v) citric acid and a filter paper soaked in 20% KOH was present in the centre well to collect the $^{14}CO_2$ produced. At the end of the incubation period the acids were tipped from the side arm to stop the reaction and to liberate any CO_2 dissolved in the medium. After a further incubation, lasting 30 min., the alkali-soaked filter papers were removed, with washings, from the centre well and extracted with carrier Na_2CO_3 ($\equiv$ 10·5 mg. of $BaCO_3$). The $^{14}CO_2$ was precipitated as $BaCO_3$ with $BaCl_2$ and the radioactivity was measured as described above, a correction for self-absorption to infinite thinness being made according to the method of Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). New alkali-soaked filter papers were placed in the centre wells and 1·0 ml. of aniline citrate (Quastel & Wheatley, 1933, as modified by Edson, 1935) was placed in the side arm. The vessels were returned to the bath and aniline citrate was tipped into the main compartment to decompose the acetoacetate present to acetone and CO_2. The $^{14}CO_2$ produced was assayed as before. The slices were then removed, washed twice with 5 ml. of cold Krebs–Ringer solution, homogenized with 5 ml. of 95% ethanol and extracted at 50° for 30 min. The residue was extracted again with 5 ml. of ethanol–ether (1:1) for 30 min. at 40°. The supernatants were mixed and the phospholipids quantitatively precipitated and purified according to the method of Sinclair & Dolan (1942). The precipitated phospholipids were dissolved in a few drops of moist ether, plated on aluminum disks and dried, and their radioactivity was counted as described before.

RESULTS

Effects of fatty acids on the respiration of tissue slices

Potassium decanoate, at concentrations of up to 1·34 mM, stimulated the oxygen uptake of rat-liver slices respiring in Krebs–Ringer solution. When the concentration of decanoate was increased to 2·67 and 4·0 mM, an initial stimulation followed by a gradual decline in the respiratory activity was observed (Fig. 1). The presence of other substrates in the medium, such as glucose, succinate, fumarate or lactate, did not alter the inhibitory effect of decanoate although the presence of the second substrate caused further stimulation of respiration when lower concentrations of fatty acid were used.

Table 1. *Effects of various fatty acids on the endogenous respiration of rat-liver slices*

All figures refer to $-Q_{O_2}$ values between the time intervals indicated. Fatty acids with chain length above C_{13} were apparently partially in suspension in the medium. All the other fatty acids were in solution at the concentration used.

Fatty acid added (4 mM) ...	None	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}
Period (min.)												
0–30	8·8	11·1	10·2	9·8	10·9	9·6	11·1	9·0	7·9	7·7	9·9	8·8
30–60	7·2	8·9	7·6	8·9	9·8	5·4	9·6	5·5	7·8	7·6	7·5	6·3
60–90	7·4	10·2	8·5	8·2	9·6	3·5	9·8	3·6	6·5	6·0	6·4	5·8
90–120	6·9	9·3	8·3	6·5	9·0	2·6	7·7	3·1	6·4	6·1	6·1	5·3

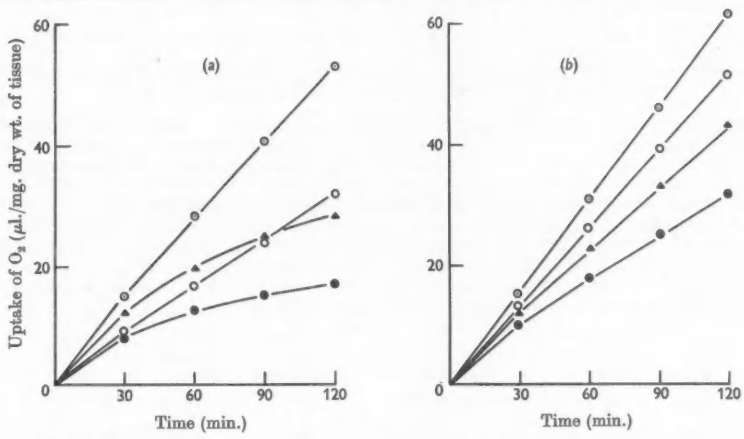

Fig. 2. Effects of potassium decanoate on the respiratory activity of rat-kidney-cortex slices (*a*) in the absence, and (*b*) in the presence, of 10 mM-sodium pyruvate. ○, No decanoate added; ⊙, 1 mM-decanoate; ▲, 1·67 mM-decanoate; ●, 2 mM-decanoate.

Fatty acids such as hexanoate, nonanoate or undecanoate, at concentrations of up to 4 mM, showed no inhibitory effect. Laurate was as effective as decanoate but higher fatty acids had little inhibitory effect (Table 1). Decanoate also inhibited the respiration of rat-kidney-cortex slices, both in the presence and absence of pyruvate, though slightly higher concentrations of the fatty acid were required to obtain inhibitions of respiration in the presence of added pyruvate (Fig. 2). The inhibitory effects on respiration increased with chain length of the fatty acid in a fashion similar to that noted above with liver slices, though with kidney slices the odd-numbered fatty acids seemed to be as effective as the even-numbered fatty acids of similar chain lengths.

Effect of decanoate on oxidative phosphorylation in rat-liver mitochondria

A concentration of 1·0–1·3 mM-decanoate was required to produce 50 % decrease of the P/O ratio for glutamate oxidation by liver mitochondria (Table 2). Scholefield (1956) observed that in brain mitochondria approximately 0·6 mM-decanoate gave 50 % decrease of the P/O ratio for pyruvate oxidation. The difference in the sensitivity of different types of mitochondria to the inhibitory effects of decanoate appeared to be related to the ability of different tissues to oxidize the fatty acid. In those tissues where the rate of oxidation of fatty acids is low, decanoate was more effective as an inhibitor.

The effect of decanoate (1·0 mM) on the oxidation of ascorbate and succinate and on the efficiency of phosphorylation was tested. The mitochondrial preparation used to measure oxidative phosphorylation with succinate as substrate was first incubated with phosphate buffer to remove endogenous diphosphopyridine nucleotide (Hunter & Ford, 1955). This treatment prevents further oxidation of the malate formed from succinate but also, in our experience, reduces the phosphorylation quotient with succinate to about one-half of that normally obtained. The addition of decanoate (1·0 mM) to this preparation had no effect on the oxygen uptake, but completely abolished the residual phosphorylation (Table 3). Similar results were obtained when ascorbate was used as substrate (Table 3). Further evidence on the similarity in action of fatty acids and dinitrophenol (DNP) was obtained by comparing their effects on the respiration of mitochondria in phosphate-deficient media. Both decanoate and DNP markedly stimulated the respiration under these conditions though the respiration in the presence of the higher concentration of decanoate gradually decreased during the incubation (Table 4).

Table 2. *Effects of decanoate on the* P/O *ratio for glutamate oxidation by rat-liver mitochondria*

Incubation was carried out for 15 min. at 37° in the medium described by Judah & Rees (1953). Glutamate (10 mM) was the substrate. Mitochondria used were equivalent to 500 mg. original wet wt. of liver. Figures in parentheses refer to percentage inhibitions of the P/O ratio.

Concn. of decanoate (mM)	Uptake of O_2 (μg.atoms)	Uptake of phosphate (μmoles)	P/O
0	10·2	25·8	2·52
0·34	11·5	23·3	2·09 (17)
0·67	13·6	19·8	1·45 (41)
1·0	13·2	18·4	1·39 (44)
1·34	13·4	7·7	0·58 (75)

Table 3. *Effects of potassium decanoate on the oxidative phosphorylation associated with cytochrome* c *oxidase and the one-step oxidation of succinate*

In Expt. 1, mitochondria were suspended in 0·075 M-sucrose and were equivalent to 500 mg. wet wt. of liver. Cytochrome *c* was reduced by 10 mM-ascorbate. 0·3 mM-Ethylenediaminetetra-acetic acid was also present. Incubation was carried out for 15 min. at 37° in the medium described in the Materials and Methods section.

In Expt. 2, 6 ml. of the standard rat-liver mitochondrial suspension in 0·25 M-sucrose was treated with 1·5 ml. of 0·1 M-phosphate buffer, pH 7·4, and allowed to stand for 10 min. at 37° as described by Hunter & Ford (1955). A portion (0·5 ml.) of this suspension was used in each vessel. This procedure depletes the mitochondria of endogenous diphosphopyridine nucleotide and thus permits succinate oxidation to proceed only as far as malate. Incubation time was 15 min. at 37° in the medium described in the Materials and Methods section.

Figures in parentheses refer to percentage inhibitions of the P/O ratio.

Expt. no.	Concn. of decanoate (mM)	Substrate	Uptake of O_2 (μg.atoms)	Uptake of phosphate (μmoles)	P/O
1	0	Ferrocytochrome *c*	12·4	6·4	0·52
	0·33		12·3	4·5	0·37 (29)
	1·0		13·0	0	0 (100)
2	0	16·7 mM-Succinate	17·6	16·8	0·96
	0·33		16·3	4·5	0·28 (72)
	1·0		14·2	0	0 (100)

Incorporation of [^{32}P]phosphate into rat-liver slices

In order to ascertain whether uncoupling of oxidation from phosphorylation occurred in intact tissues, the effects of fatty acids on [^{32}P]phosphate incorporation in various phosphate fractions of liver slices were also investigated.

Time course. When rat-liver slices were incubated with [^{32}P]phosphate, its incorporation into the inorganic phosphate fraction and the ATP plus ADP fraction was very rapid. Most of the labelling occurred within 20 min. and little increase in labelling occurred during the subsequent 60 min. of incubation (Table 5). The labelling of the total phospholipid fraction continued to increase during the entire incubation period whereas in the acid-insoluble fraction (phosphoprotein, nucleic acids etc.) an equilibrium was attained within 40 min. Glucose did not stimulate incorporation of [^{32}P]-phosphate.

Table 4. *Effects of decanoate on the respiration of rat-liver mitochondria in a phosphate-deficient medium*

All values are for μl. of oxygen taken up. Experimental conditions were as described in Table 2. Incubation medium was changed by omission of glucose and hexokinase and replacement of 10 mM-phosphate by 1·67 mM-phosphate plus 67 mM-2-amino-2-hydroxymethylpropane-1:3-diol, pH 7·4.

	Additions			
	Nil	Decanoate		DNP
Time (min.)		0·34 mM	0·67 mM	0·05 mM
5	9	24	47	34
10	18	40	79	72
15	32	55	100	110
20	56	77	116	150
25	82	107	136	186

Effect of potassium decanoate on incorporation of [^{32}P]phosphate. Potassium decanoate at concentrations of 0·67, 1·34 and 2·0 mM had no inhibitory effect on the respiratory activity of liver slices and little effect on the incorporation of ^{32}P into the inorganic phosphate but strongly inhibited [^{32}P]-phosphate incorporation into the ATP plus ADP, phospholipids and acid-insoluble fraction (Table 6). A time-course study of the effect of 4 mM-decanoate (which initially stimulated respiration but gradually produced an inhibition) revealed that the [^{32}P]phosphate incorporation into the organic phosphate compounds was completely inhibited (Fig. 3) and that no labelling occurred even after prolonged incubation.

Effect of homologous fatty acids. The effect of fatty acids with chain lengths of from four to eight carbon atoms (used at a concentration of 4 mM) and with chain length of from eight to twelve carbon atoms (used at a concentration of 1·34 mM) on [^{32}P]phosphate incorporation was also studied (Table 7). At this concentration the fatty acids only stimulated respiration. Butyrate slightly inhibited phosphate incorporation into the various organic phosphate fractions but the effect increased with chain length and octanoate was the most inhibitory in this series, producing almost complete inhibition. The fatty acids of chain length C_8–C_{12} were much more inhibitory and 1·34 mM-dodecanoate completely inhibited phosphate incorporation into the organic phosphate compounds. Octanoate was the weakest inhibitor in this series and the odd-numbered fatty acids were less effective than either of the adjacent even-numbered fatty acids. The above results bore close resemblance to those obtained with DNP, which also inhibited phosphate incorporation into organic phosphate compounds under conditions where it had little inhibitory effect on respiratory activity of the slices (Table 8).

Table 5. *Time course of incorporation of [^{32}P]phosphate into various phosphate compounds in rat-liver slices*

^{32}P was tipped into the main compartment of Warburg vessels at zero time and the incubation was carried out at 37°. The basic incubation medium used was a calcium-free Krebs–Ringer phosphate solution as described in the Materials and Methods section.

		Phosphate incorporated (μm-moles/100 mg. wet wt. of tissue)			
Time (min.)	Glucose (10 mM)	P_i	ATP plus ADP	Phospholipids	Acid-insoluble fraction (phosphoproteins, RNA and DNA)
20	+	347	123	10	12
	−	329	128	8	16
40	+	366	125	13	27
	−	381	136	14	29
60	+	404	142	21	33
	−	416	152	19	33
80	+	394	111	23	27
	−	395	126	24	34

Metabolism of [1-^{14}C]*decanoate*

The metabolism of [1-^{14}C]decanoate, at several concentrations, to $^{14}CO_2$, to [^{14}C]acetoacetate and to ^{14}C-labelled phospholipids was studied (Table 9). The amount of $^{14}CO_2$ released by aniline citrate from the acetoacetate formed during incubation increased with increasing concentrations of [1-^{14}C]-decanoate, although less $^{14}CO_2$ was obtained when the incubation was carried out at concentrations higher than 1·33 mM. Thus the acetoacetate produced from 2·67 mM-decanoate was only slightly more than that produced in the presence of 0·33 mM.

The incorporation of the ^{14}C into phospholipids continued at an almost constant rate even in the presence of 2 mM-decanoate, but with 2·67 mM the rate of incorporation decreased slightly with time. Thus decanoate, at concentrations which strongly inhibit phosphate incorporation but do not inhibit the respiratory activity of the tissue, has little effect on its own oxidation or on its incorporation into phospholipids.

The results from respiratory studies showed that the inhibitory effects of fatty acids at higher concentrations set in gradually and only after an initial stimulatory phase. To determine if this pattern coincided with the pattern of fatty acid oxidation, a time-course study of the oxidation of 1·34 and 4 mM-decanoate was undertaken. The results presented in Fig. 4 demonstrate that with 1·34 mM-decanoate, the rates of respiration, $^{14}CO_2$ production and acetoacetate production were relatively constant during an incubation period lasting 200 min. There was an initial increase in respiratory activity in the presence of 4 mM-decanoate but it declined considerably at the end of 60 min. The initial rate of $^{14}CO_2$ production was also slightly higher than that from 1·34 mM-decanoate, but it completely ceased after 80 min. and production of $^{14}CO_2$ from acetoacetate ceased after 50 min.

Table 6. *Effect of various concentrations of decanoate on incorporation of* [^{32}P]*phosphate into rat-liver slices*

Decanoate was present in the main vessel and ^{32}P was tipped in at zero time. Incubation was carried out for 60 min. at 37°. No added substrate was present and the slices were respiring in a calcium-free Krebs–Ringer phosphate solution.

Concn. of decanoate (mM)	Phosphate incorporated (μm-moles/100 mg. wet wt. of tissue)			
	P_i	ATP plus ADP	Phospho-lipids	Acid-insoluble fraction
0	496	123	30	40
0·67	503	51	18	24
1·34	398	29	8	9
2·00	379	6	4	6

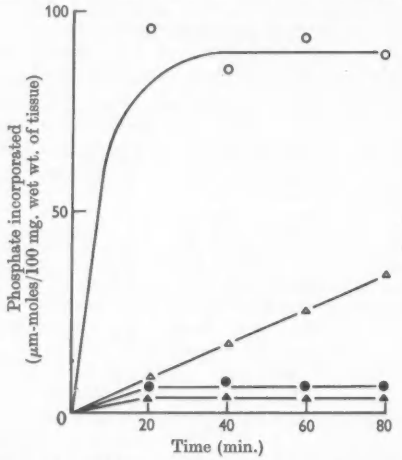

Fig. 3. Effects of 4 mM-potassium decanoate on phosphate incorporation into rat-liver slices. ○, △, No decanoate added; ●, ▲, 4 mM-decanoate added. ○, ●, Incorporation into ATP plus ADP; △, ▲, incorporation into phospholipids.

Table 7. *Effect of various fatty acids on incorporation of* [^{32}P]*phosphate into various fractions of rat-liver slices*

Experimental conditions were the same as described for Table 6.

Fatty acid			Phosphate incorporated (μm-moles/100 mg. wet wt. of tissue)		
Chain length	Concn. (mM)	$-Q_{O_2}$	P_i	ATP plus ADP	Phospholipids
—	0	8·3	655	154	68
4	4·0	8·6	654	141	53
5	4·0	8·8	674	102	46
6	4·0	10·6	727	100	44
7	4·0	9·7	624	80	32
8	4·0	8·9	632	38	13
—	0	8·0	495	125	40
8	1·34	8·1	566	61	27
9	1·34	9·2	489	89	27
10	1·34	8·6	535	30	11
11	1·34	9·6	496	60	17
12	1·34	6·5	471	11	6

DISCUSSION

The present experiments on the inhibitory effects of fatty acids on the respiratory activity of rat-liver slices have confirmed earlier observations made with guinea-pig-liver slices (Quastel & Wheatley, 1933) and mitochondrial preparations (Lehninger, 1951; Scholefield, 1956). Among liver, kidney and brain tissues, liver is the least sensitive to the inhibitory effects of fatty acids on respiration and brain tissue the most sensitive (Ahmed & Scholefield, 1961), which is in keeping with the poor ability of brain tissue to oxidize fatty acids. In addition, a concentration of 1·0–1·3 mM-decanoate is required to produce a 50% decrease in the P/O ratio for glutamate oxidation in rat-liver mitochondria, whereas 0·6 mM produces a similar effect in rat-brain mitochondria (Scholefield, 1956). It may be noted that the phosphorylations associated with isolated enzyme systems such as cytochrome *c* oxidase and succinate oxidation via cytochrome *c* oxidase are even more sensitive to decanoate. These results show that the presence of fatty acids gives rise to an inhibition of the various phosphorylations associated with the respiratory chain and suggests that the inhibitory effects are due to the presence of free fatty acids themselves.

Table 8. *Effect of dinitrophenol on incorporation of [^{32}P]phosphate into rat-liver slices*

Experimental conditions were the same as described for Table 6.

Concn. of DNP (mM)	Phosphate incorporated (μm-moles/100 mg. wet wt. of tissue)			
	P_i	ATP plus ADP	Phospho-lipids	Acid-insoluble fraction
0	422	150	37	46
0·025	513	76	23	40
0·075	378	6	10	21
0·150	284	0	6	10

Table 9. *Oxidation and incorporation of [1-^{14}C] decanoate by rat-liver slices*

[1-^{14}C]Decanoate had the same specific activity throughout (0·2 μc/μmole). Incubation was carried out for 60 min. at 37°. No added substrate was present in the medium, which was a calcium-free Krebs–Ringer phosphate solution.

Concn. of [1-^{14}C]decanoate (mM)	$-Q_{O_2}$	Decanoate oxidized (μm-moles/mg. dry wt./hr.)	Acetoacetate $^{14}CO_2$ (μm-moles/mg. dry wt./hr.)	[1-^{14}C]Decanoate incorporated into phospholipids (μm-moles/100 mg. wet wt. of tissue/hr.)
0	7·6	—	—	—
0·33	8·9	10·1	10·6	1·34
1·0	9·8	12·4	22·0	4·0
1·33	9·5	14·1	25·2	4·0
2·00	8·8	12·3	22·6	6·3
2·67	9·3	15·6	13·7	8·0

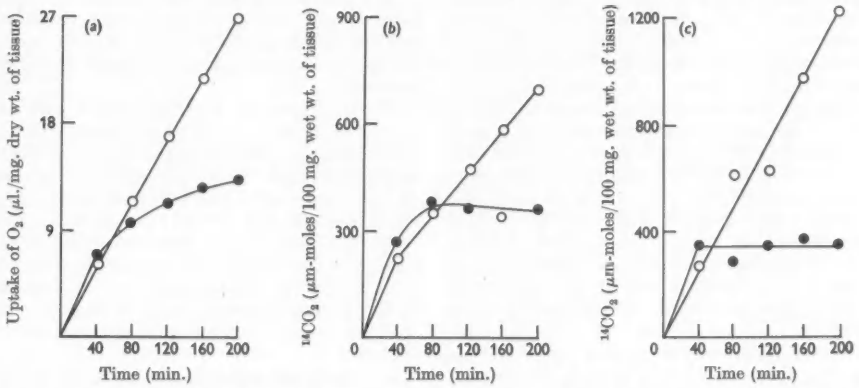

Fig. 4. Effects of potassium decanoate on (*a*) respiratory activity of rat-liver slices, (*b*) oxidation of [1-^{14}C]decanoate to $^{14}CO_2$ and (*c*) production of $^{14}CO_2$ from decarboxylation of acetoacetate formed during incubation. ○, 1·34 mM-[1-^{14}C]Decanoate present; ●, 4 mM-[1-^{14}C]decanoate present.

The results obtained in the study of the metabolism of [1-^{14}C]decanoate leave no doubt that it is oxidized and also incorporated into lipids during an incubation period of 1 hr. Under these conditions, enough ATP must be available for continued activation of the fatty acid since 1·34 mM-[1-^{14}C]-decanoate continued to be oxidized at a steady rate even after 3 hr. However, the oxidation of 4 mM-[1-^{14}C]decanoate to $^{14}CO_2$ stops at the end of about 80 min., which suggests that all the ATP in the cell may have then been depleted and that the process of oxidative phosphorylation may have been completely inhibited. The respiratory activity of the slice also falls to a low level. With 4 mM-[1-^{14}C]-decanoate a small amount of the acid may initially be transported into the cell, where some of it is activated and oxidized, but as it accumulates inside the cell it eventually causes an inhibition of the ATP-regenerating mechanism.

On the other hand, incorporation of [^{32}P]phosphate into liver slices is strongly inhibited by fatty acids at concentrations at which their own oxidation and incorporation into lipids proceeds at a high rate and at which they are not inhibitory to respiration. A satisfactory explanation of this effect may be offered in terms of the ATP–$^{32}P_i$ exchange reaction in liver mitochondria (Ahmed & Scholefield, 1960). This exchange proceeds very rapidly in isolated liver mitochondria and is sensitive to fatty acids (0·2 mM-decanoate inhibited the exchange reaction by about 50%, compared with the concentration of 1·0–1·3 mM required for a similar inhibition of oxidative phosphorylation in rat-liver mitochondria). A large part of the labelling of ATP with ^{32}P (and hence of other organic phosphates whose synthesis is dependent upon ATP) in intact slices must therefore take place through the participation of this reaction rather than by true synthesis of ATP, and it must be the exchange reaction which is inhibited on addition of relatively low concentrations of decanoate (and other fatty acids) to rat-liver slices. It is probable that the exchange reaction is also of importance *in vivo*, for Kaplan & Greenberg (1944) have reported a rapid equilibration of $^{32}P_i$ with the ATP of liver *in vivo*.

It was observed by Dianzani (1954) that mitochondria prepared from fatty livers showed a low P/O ratio. He suggested that the loss of phosphorylative ability in fatty livers may be a consequence of morphological changes which had led to degenerative processes *in vivo*. It is possible that the development of fatty livers leading to steatosis, or complete liver degeneration, is a manifestation of the uncoupling action of fatty acids *in vivo*. Similar considerations might apply to the deposition of excess of lipids in other tissues, such as occurs, for example, in arteries during arteriosclerosis.

Lehninger & Remmert (1959) have shown that 3 μM-oleate produces swelling of mitochondria. In addition to inhibitory effects on ATP–$^{32}P_i$ exchange, fatty acids at still lower concentrations considerably stimulate the exchange reaction, an effect which may be considered to be a consequence of changed permeability of mitochondria in the presence of added fatty acids (Ahmed & Scholefield, 1960). This increased permeability of mitochondria might cause a loss of cofactors, such as DPN, and eventually lead to the observed loss of respiratory activity. These considerations suggest that the mechanism of action of fatty acids is somewhat different from that of DNP, which generally does not cause swelling of mitochondria (Chappell & Greville, 1958; Lehninger, 1959).

Several workers (Pullman & Racker, 1956; Polis & Schmukler, 1957; Lehninger & Remmert, 1959) have reported the presence of endogenous uncoupling factors in mitochondria and it is likely that some of these factors resemble fatty acids such as oleic acid (Lehninger & Remmert, 1959). Of the saturated fatty acids, tridecanoate had the greatest inhibitory effect on the ATP–^{32}P exchange in mitochondria (Ahmed & Scholefield, 1960) and dodecanoate the greatest effect on respiration of slices but oleic acid was more effective than any of the saturated fatty acids in inhibiting the ATP–^{32}P exchange, e.g. 2 μM-oleate inhibited this reaction by about 50% (K. Ahmed & P. G. Scholefield, unpublished data). The unsaturated fatty acids and the medium-chain-length saturated fatty acids may thus have some role as normal intracellular or endogenous swelling and uncoupling agents.

SUMMARY

1. Fatty acids increase the respiratory activity of rat-liver and -kidney slices. At higher concentrations of the fatty acids, the initial stimulation of the rate of oxygen uptake is followed by a gradual decrease.

2. The inhibitory effects increase with chain length, dodecanoate having the greatest effect. The inhibitions produced by higher fatty acids were not as great. Fatty acids containing an odd number of carbon atoms were less effective inhibitors than those with one more or one less carbon atom.

3. Decanoate (1 mM) inhibited the oxidative phosphorylation associated with glutamate oxidation in rat-liver mitochondria by approximately 50%. It also decreased the P/O ratio associated with the oxidation of succinate and reduced cytochrome *c*.

4. Decanoate and other fatty acids inhibited incorporation of [^{32}P]phosphate into rat-liver slices at a concentration (1·34 mM) which caused a stimulation of respiratory activity. [1-^{14}C]Decano-

ate was oxidized to $^{14}CO_2$ and incorporated into lipids at these concentrations.

5. The inhibitory effects of fatty acids are explained in terms of their effects on the enzyme systems involved in oxidative phosphorylation.

It is a pleasure to thank Professor J. H. Quastel, F.R.S., for his continued interest in this work and to acknowledge the financial support of the National Cancer Institute of Canada. One of us (K. A.) was the holder of a Colombo Plan Fellowship under the Technical Assistance Programme by the Government of Canada, Department of Trade and Commerce, to the Republic of Pakistan.

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Studies on Fatty Acid Oxidation

8. THE EFFECTS OF FATTY ACIDS ON METABOLISM OF RAT-BRAIN CORTEX *IN VITRO*

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In the preceding paper (Ahmed & Scholefield, 1961) it was shown that fatty acids uncouple oxidation from phosphorylation in rat liver. The present studies were undertaken to survey the effects of fatty acids on various aspects of brain metabolism *in vitro*. These studies were of interest in view of the observation by Samson & Dahl (1955) that injection of short-chain fatty acids produces narcotic-like effects in normal animals.

It was previously demonstrated that the rapid exchange reaction between adenosine triphosphate and ^{32}P-labelled phosphate brought about by rat-liver mitochondria is a significant factor in the labelling of adenosine triphosphate and other organic phosphates in rat-liver slices (Ahmed & Scholefield, 1960). In the present paper the conditions necessary for measurement of the rate of the adenosine triphosphate–inorganic [^{32}P]phosphate (ATP–$^{32}P_i$) exchange reaction in rat-brain mitochondria have been examined. Findlay, Rossiter & Strickland (1953) observed that glucose and oxygen were essential for the incorporation of ^{32}P into the nucleic acid fraction of cat-brain slices. This labelling was markedly decreased when the members of the tricarboxylic acid cycle were used

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as substrates in place of glucose and also when conditions were made anaerobic. The effects of decanoate and dinitrophenol on the incorporation of ^{32}P into various fractions of rat-brain-cortex slices have now been investigated to determine the part played by synthesis of adenosine triphosphate in the labelling of the various organic phosphate fractions.

MATERIALS AND METHODS

Animals. Adult hooded rats of a local inbred strain, weighing approx. 200 g., were used throughout this investigation.

Chemicals. All common chemicals were of reagent grade and were used without further purification. Adenosine triphosphate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; cytochrome *c* from Wyeth Inc., Philadelphia, Pa., U.S.A.; and hexokinase from the Sigma Chemical Co., St Louis, Mo., U.S.A. Radioactive phosphate was purchased from Charles E. Frost and Co., Montreal, P.Q., Canada. It was purified as described by Ahmed & Scholefield (1961). All the solutions and experimental media were adjusted to pH 7·4.

Tissue preparations. Rat-brain mitochondria were prepared according to Brody & Bain (1952) by centrifuging rat-brain homogenates in 0·25 M-sucrose containing 1 mM-ethylenediaminetetra-acetate (EDTA), pH 7·4, for 10 min. at 1200*g* to remove nuclei and cell debris, and then at 10 500*g* for 10 min. to obtain the mitochondrial pellet, which was washed once with 0·25 M-sucrose solution. All these manipulations were carried out at 2°.

Rat brains were removed and chilled in ice-cold Krebs–Ringer salts solution. Cortex slices were cut from cerebral hemispheres by means of a Stadie–Riggs tissue slicer (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.).

Incubation techniques. Oxygen uptake was measured with the conventional Warburg apparatus at 37° in an atmosphere of O_2 for slices and in air for mitochondria. For the measurement of aerobic glycolysis $O_2 + CO_2$ (93:7) was used and for anaerobic glycolysis $N_2 + CO_2$ (93:7) was used as gas phase.

The Krebs–Ringer medium used for incubation had the following composition: NaCl, 145 mM; KCl, 5·8 mM; KH_2PO_4, 1·5 mM; $MgSO_4$, 1·5 mM; $CaCl_2$, 3·1 mM and sodium phosphate buffer, pH 7·4, 10 mM, the final pH being 7·3. The volume of the incubation medium was 3 ml. in all cases. When aerobic or anaerobic glycolysis was measured, the phosphate buffer was replaced by 25 mM-$NaHCO_3$ to yield a final pH of 7·4. The media used for incubation of mitochondria are as described in the text.

Estimation of radioactivity. The methods used in assaying [^{32}P]phosphate incorporation into slices and the ATP–$^{32}P_i$ exchange in mitochondria are as described in the preceding paper (Ahmed & Scholefield, 1961). All counting was done to an accuracy of within 5%.

Calculation of results. Most of the results are expressed in terms of μm-moles of phosphate incorporated into the various fractions/100 mg. wet wt. of tissue, calculated from the formula given in the preceding paper.

All experiments were performed at least twice and either typical or mean values are quoted.

RESULTS

Effects of decanoate and other fatty acids on the respiratory activity of rat-brain-cortex slices

Time course of respiration. Brain oxidizes fatty acids very slowly. When low concentrations of fatty acids are added to brain slices respiring in the presence of glucose, a slight stimulation of the rate of oxygen uptake is observed, but increase in the fatty acid concentration leads to the cessation of respiratory activity after an initial stimulatory phase. Thus 0·25 mM-decanoate stimulated respiration throughout an incubation period of 120 min. but when 0·5 mM-decanoate was used there was an initial stimulation and after incubation for 90 min. the rate of respiration fell off markedly (Fig. 1). A similar phenomenon was observed when 0·75 mM-decanoate was used but the decrease in the rate of oxygen uptake occurred after incubation for 60 min. (Fig. 1). When 100 mM-KCl was added to the incubation medium, a stimulation of respiratory activity was observed both in the presence and in the absence of 0·5 mM-decanoate, but the 'fall-off' of the respiratory activity in the former occurred after 60 min. incubation compared with 90 min. for brain cortex incubated with the standard amount of potassium (7·3 mM).

All the fatty acids tested, from heptanoate to myristate, produced inhibitory effects at higher concentrations. In Fig. 2 is shown the relationship between chain length of the fatty acids and the concentrations required to produce a 'fall-off' of

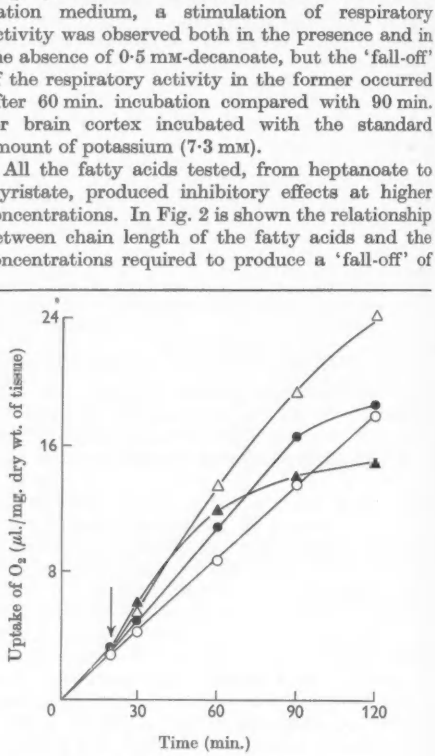

Fig. 1. Effects of 0·5 mM-potassium decanoate and 100 mM-KCl on the respiratory activity of rat-brain-cortex slices in the presence of 10 mM-glucose. Additions made at 20 min.: ○, nil; ●, decanoate; △, KCl; ▲, decanoate plus KCl.

respiratory activity in 45–60 min. Heptanoate produced such an effect at a concentration of 9–10 mM, but dodecanoate, the most effective inhibitor in the series, caused the same effect at a concentration of 0·2 mM. Fatty acids with chain length greater than dodecanoate were found to be relatively less effective.

Effect of washing the slices after treatment with fatty acids. Rat-brain-cortex slices were incubated in the presence of glucose and 0·8 mM-decanoate for various time intervals. They were then removed from the incubation medium, washed with cold Krebs–Ringer solution and incubated in fresh medium containing glucose but no added decanoate. The slices which had been subjected to 20 min. preincubation maintained their respiratory activity in the new medium (Fig. 3). However, slices which had been preincubated with 0·8 mM-decanoate for 60 min. respired at lower rates than did the controls. Thus some form of irreversible damage had occurred.

Effects of decanoate in the presence of various substrates. The effect of decanoate on the respiration of brain-cortex slices in the presence of several carboxylic acids was studied and compared with the effect obtained with glucose as the substrate (Fig. 4). The oxidation of succinate was insensitive to 0·8 mM-decanoate. The respiration in the presence of other substrates, such as fumarate, glutamate, α-oxoglutarate and pyruvate, was decreased by decanoate but not to the same extent as was the respiration in the presence of glucose.

Effects of fatty acids on aerobic and anaerobic glycolysis

A comparison of the effects of potassium decanoate and dinitrophenol (DNP) on respiration, aerobic glycolysis and anaerobic glycolysis in rat-brain-cortex slices is shown in Fig. 5. Decanoate

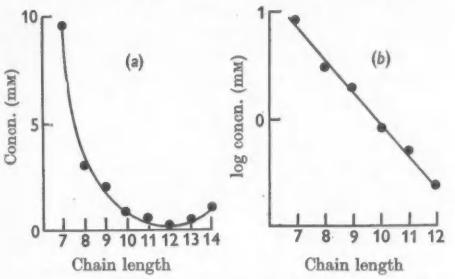

Fig. 2. Relation between chain length of fatty acid and (*a*) concentration or (*b*) logarithm of the concentration required to produce a 'fall-off' in respiratory activity of rat-brain-cortex slices in the presence of 10 mM-glucose in 45–60 min.

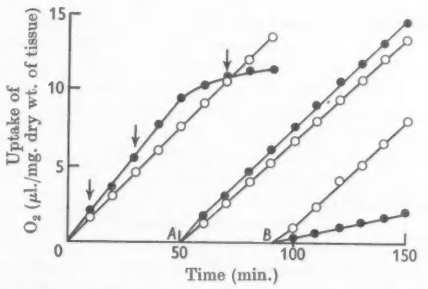

Fig. 3. Effects of washing slices and transferring them to new media after preincubation with 10 mM-glucose and 0·8 mM-potassium decanoate (●) or with 10 mM-glucose only (○). Six Warburg vessels were set up, three with and three without decanoate in the side arms. After incubation for 10 min. (first arrow) decanoate was tipped into the main compartment of the appropriate vessels. After incubation for a further 20 min. one pair of vessels was removed (second arrow); the slices were washed and incubated in fresh media containing 10 mM-glucose but no decanoate. Subsequent respiratory activity is shown in the pair of curves commencing at point *A*. After incubation for a further 40 min., another pair of vessels was removed (third arrow), the slices were washed and incubated as before in fresh media containing 10 mM-glucose but no decanoate. Subsequent respiratory activity is shown in the pair of curves commencing at point *B*. All incubations were carried out at 37° in a Krebs–Ringer phosphate medium.

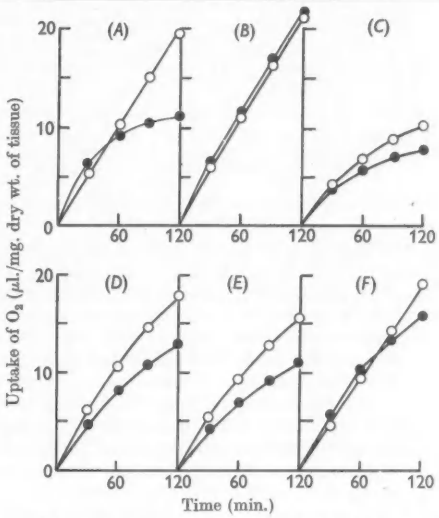

Fig. 4. Effects of 0·8 mM-decanoate on the respiration of rat-brain-cortex slices in the presence of various substrates (each 10 mM). Decanoate added: ○, nil; ●, 0·8 mM. (*A*) Glucose; (*B*) succinate; (*C*) fumarate; (*D*) glutamate; (*E*) α-oxoglutarate; (*F*) pyruvate.

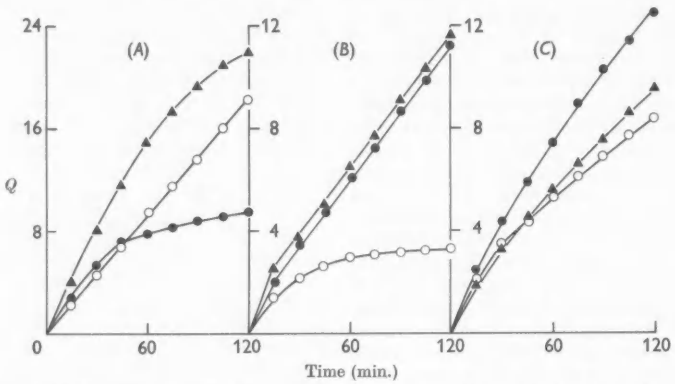

Fig. 5. Effects of 0·8 mM-decanoate and 0·06 mM-DNP on respiration (*A*), aerobic glycolysis (*B*) and anaerobic glycolysis (*C*), by rat-brain-cortex slices. Additions: ○, nil; ●, 0·8 mM-decanoate; ▲, 0·06 mM-DNP. Glucose (10 mM) was present in all vessels. *Q* values refer in (*A*) to μl. of oxygen uptake, and in (*B*) and (*C*) to μl. of CO_2 evolved/mg. dry wt. of tissue.

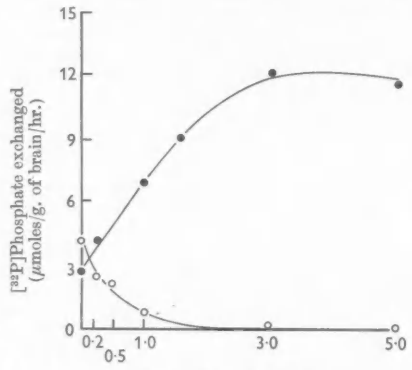

Fig. 6. Effects of Mg^{2+} ions on the ATP–$^{32}P_i$ exchange reaction in rat-brain mitochondria in the presence and absence of 10 mM-EDTA. ○, No EDTA present; ●, 10 mM-EDTA added.

(0·8 mM) slightly stimulated anaerobic glycolysis and DNP (0·06 mM) gave a similar result. Aerobic glycolysis in normal slices was low and stopped completely within 60 min. When 0·8 mM-decanoate or 0·06 mM-DNP was added to the incubation medium, the rate of aerobic glycolysis approached that of anaerobic glycolysis.

Effects of decanoate on the adenosine triphosphate–$^{32}P_i$ exchange reaction in rat-brain mitochondria

The conditions required for the measurement of the ATP–$^{32}P_i$ exchange in rat-brain mitochondria were found to be somewhat different from those reported for liver mitochondria. When rat-brain mitochondria were isolated from 0·25 M-sucrose solution containing 1 mM-ethylenediaminetetraacetic acid (EDTA), they could bring about oxidative phosphorylation at 37° or 20°, but no exchange reaction could be demonstrated when the incubation was carried out under similar conditions at 20°. This apparent absence of the exchange reaction could not be accounted for by loss of ATP through adenosine-triphosphatase activity since analysis indicated little if any accumulation of inorganic phosphate. Further experiments showed that the occurrence of the ATP–$^{32}P_i$ exchange reaction in these mitochondria could be demonstrated by decreasing the magnesium content of the medium or by adding 10 mM-EDTA (Fig. 6). The complete omission of Mg^{2+} ions from the medium, however, produced variable results, and experience showed that the most consistent results were obtained when the medium contained both 5 mM-$MgSO_4$ and 10 mM-EDTA. The labelling of ATP increased linearly with time when mitochondria equivalent to 300 mg. original wet wt. of tissue were incubated for 15 min. at 20°. The calculated rate of the ATP–$^{32}P_i$ exchange reaction in rat-brain mitochondria was 14 μmoles of [^{32}P]-phosphate exchanged/g. wet wt. of brain/hr. The corresponding value found for liver was 84 μmoles (Ahmed & Scholefield, 1960).

Another major difference between liver and brain mitochondria is their sensitivity towards inhibitors of the exchange reaction, such as DNP and decanoate. DNP (13·5 μM) inhibited the exchange reaction in brain by about 75 % (Table 1), whereas in

Table 1. *Effect of decanoate and dinitrophenol on the adenosine triphosphate–$^{32}P_i$ exchange reaction in rat-brain mitochondria*

Values in parentheses refer to percentage of the control in each case. The incubation medium contained: ATP, 2·5 mM; $MgSO_4$, 5 mM; KCl, 37·5 mM; tris buffer, pH 7·4, 100 mM; EDTA, 10 mM; sodium phosphate buffer, pH 7·4, 5 mM, and ^{32}P, 1–2 μc. Gas phase, air. Mitochondria equivalent to 125 mg. wet wt. of brain were added to start the reaction at 20°. Incubation time was 30 min.

Inhibitor	Concn. of inhibitor (mM)	Phosphate exchanged (μmoles/hr./g. wet wt. of tissue)
Decanoate	0	14·0 (100)
	0·1	14·2 (102)
	0·2	12·3 (88)
	0·3	8·9 (64)
	0·4	7·8 (56)
DNP	0	13·4 (100)
	0·0045	9·3 (69)
	0·009	6·9 (52)
	0·0135	3·6 (27)

liver mitochondria a comparable inhibition of the exchange may be observed in the presence of 5 μM-DNP. No significant increase in the rate of the ATP–$^{32}P_i$ exchange reaction in brain mitochondria could be observed in the presence of very low concentrations of decanoate such as occurs in liver mitochondria (Ahmed & Scholefield, 1960). The concentration of decanoate required to produce a 50% inhibition of the exchange reaction, being about 0·4 mM, is of the same order as that required to produce a 50% inhibition of oxidative phosphorylation in brain mitochondria (Scholefield, 1956).

Effects of decanoate on incorporation of ^{32}P into brain-cortex slices

Time course of ^{32}P incorporation. The labelling of organic phosphate compounds with ^{32}P in brain slices with glucose as substrate takes place very slowly. In order to increase the intensity of labelling, the specific activity of the phosphate was increased by reducing the concentration of carrier inorganic phosphate in the incubation medium from 10 to 3·3 mM. The uptake of phosphate from the medium containing 3·3 mM-phosphate was about one-third of that obtained in the presence of 10 mM-phosphate (Table 2). The labelling of this fraction and of the organic phosphates continued to increase during an incubation period of 120 min. The difference in the amount of carrier phosphate did not produce a significant effect on the rate of phosphate incorporation into various organic phosphate compounds, when the latter were expressed in terms of μm-moles of phosphate/100 mg. of tissue, with the possible exception of the phospholipid and acid-insoluble fractions.

Effects of decanoate on incorporation of ^{32}P with added glucose. The effects of 0·3 and 0·6 mM-decanoate on incorporation of ^{32}P into rat-brain-cortex slices in the presence of glucose were studied. Decanoate (0·3 mM) slightly stimulated respiration during the entire incubation period whereas 0·6 mM-decanoate began to inhibit respiration after incubation for 60 min. No effect on the uptake of inorganic phosphate was observed (Table 3) but there was a marked effect on the phosphate incorporation into the organic phosphates. Decanoate (0·3 mM) inhibited incorporation of ^{32}P into the phospholipid, ATP plus ADP, acid-insoluble and 'other nucleotides' fractions by about 50% when the slices were incubated for 30 min., the inhibition being increased to about 80% at the end of incubation for 120 min. With 0·6 mM-decanoate, the incorporation of ^{32}P of all the fractions was inhibited by more than 80% within 30 min. Subsequently the rate of oxygen uptake began to decrease rapidly and there was little respiration in the presence of 0·6 mM-decanoate during the second hour of incubation. The level of radioactivity in the inorganic phosphate fraction rose but the levels in all the organic phosphate fractions fell.

Incorporation of ^{32}P into brain-cortex slices in the presence of other substrates. Incorporation of ^{32}P into various fractions was studied with glucose, succinate, glutamate, pyruvate and α-oxoglutarate as substrates (Table 4). The presence or absence of substrate appeared to have relatively little effect on the uptake of phosphate. The incorporation of phosphate, however, greatly depended upon the nature of the substrate added. Maximum incorporation of phosphate into the various organic phosphate fractions took place only in the presence of glucose and was minimal when no substrate was added. With succinate or glutamate as substrate, the phosphate incorporation into all the organic phosphate fractions, including the ATP plus ADP fraction, was of the same order as when no substrate was added. Phosphate incorporation with pyruvate or α-oxoglutarate as substrate was slightly greater than with the other substrates but was still only about 25–50% of the values obtained with glucose [see values for ATP plus ADP, phospholipids etc. (Table 4)]. The labelling of ATP, ADP and the other nucleotides was maximal only when glucose was used as substrate.

The effects of 0·6 mM-decanoate and 0·05 mM-DNP on incorporation of [^{32}P]phosphate into brain slices with succinate as substrate were also studied. As shown above, the phosphate incorporation in the presence of this substrate is very low and these inhibitors reduced it to still lower values.

Table 2. *Time course of incorporation of [^{32}P]phosphate into various fractions in rat-brain-cortex slices*

All values refer to μm-moles of phosphate incorporated/100 mg. wet wt. of brain-cortex slices. Other nucleotides included UTP, GTP and GDP. Slices were incubated at 37° in Krebs–Ringer medium containing the amounts of phosphate buffer indicated. Glucose (10 mM) was present as substrate and the gas phase was O_2.

Concn. of sodium phosphate, pH 7·4 (mM)	Incubation time (min.)	P_i	ATP plus ADP	Other nucleotides	Phospholipids	Acid-insoluble fraction
10	30	1020	28	9	11	13
	120	2790	53	22	31	29
3·3	30	360	29	6	10	7
	120	720	60	18	26	21

Table 3. *Effect of decanoate on incorporation of [^{32}P]phosphate into rat-brain-cortex slices*

All values refer to μm-moles of phosphate incorporated/100 mg. wet wt. of brain-cortex slices. All other conditions were the same as for Table 2, the concentration of phosphate being 3·3 mM.

Incubation time (min.)	Concn. of decanoate (mM)	P_i	ATP plus ADP	Phospholipids	Acid-insoluble fraction
30	Nil	261	23	7·9	6
	0·3	207	14	2·7	4
	0·6	250	2	1·5	1
120	Nil	443	63	15·4	16
	0·3	363	10	4·8	8
	0·6	325	1	0·8	1

Table 4. *Effect of various substrates on incorporation of [^{32}P]phosphate into rat-brain-cortex slices*

All values refer to μm-moles of phosphate incorporated/100 mg. wet wt. of rat-brain-cortex slices. Incubation was for 60 min. at 37° in a Krebs–Ringer medium containing 3·3 mM-phosphate buffer, pH 7·4.

Substrate (10 mM)	Total acid-soluble phosphates	P_i	Phospholipids	Acid-insoluble fraction	ATP plus ADP	Other nucleotides
Nil	376	345	3·4	4	6	2·5
Glucose	332	292	9·0	12	41	4·9
Succinate	373	360	2·6	4	6	2·5
Glutamate	343	333	2·5	5	5	2·2
Pyruvate	389	372	4·3	8	12	3·9
α-Oxoglutarate	388	369	3·8	6	13	3·4

DISCUSSION

Decanoate and other fatty acids (between C_7 and C_{14}) cause a gradual cessation of respiratory activity of brain-cortex slices incubated with glucose. The phenomenon appears to be characteristic of this tissue and was not observed with either kidney or liver slices, where inhibition of respiratory activity of tissues increases much more slowly with time. This 'fall-off' of respiration may be correlated with the effects of decanoate and DNP on glycolysis. Anaerobic glycolysis of brain slices is only slightly stimulated by these agents but the effect on aerobic glycolysis is very striking. In the absence of decanoate or DNP, brain-cortex slices exhibited a low aerobic glycolysis, which ceased completely within 45 min. In the presence of 0·8 mM-decanoate or 0·06 mM-DNP the aerobic glycolysis of rat-brain-cortex slices was greatly stimulated and remained at a high rate for more than 120 min. It is well known that uncoupling agents can reverse the Pasteur effect (Dickens, 1951) and the fact that decanoate behaves in a similar fashion is undoubtedly a consequence of its ability to act as an uncoupling agent. A stimulation of aerobic glycolysis, when slices are respiring with glucose as substrate, yields a large amount of lactic acid and a sufficient amount may accumulate inside the cell to cause a lowering of the internal pH. This would result in cellular damage and produce the 'fall-off' of respiratory activity similar to that observed in Ehrlich ascites-carcinoma cells (Scholefield, 1958). Further evidence to favour this suggestion was obtained from experiments in which slices were incubated with glucose in the presence or absence of decanoate for different periods of time

and then transferred to fresh media. The inhibition of respiration resulting from the presence of decanoate was irreversible. The preliminary incubation had thus produced permanent damage, which, in this case, was probably due to pH effects. Further, this 'fall-off' is observed only in the presence of glucose and did not occur when other substrates, which do not give rise to large amounts of acids such as lactic acid, were used. The inhibitory effects increased to a maximum with chain length C_{12}, the fatty acids having a greater number of carbon atoms being less effective. The higher fatty acids, with their greater lipid solubility, may tend to be retarded by the cell membrane and so be less effective than the fatty acids of medium chain length.

The effects of decanoate on incorporation of ^{32}P into brain-cortex slices lend support to the suggestion that the membrane may play a role in determining the effectiveness of the fatty acids. Thus 0·3 mM-decanoate had little effect on brain respiration during the course of an incubation period of 2 hr. However, it gave rise to about 50% inhibition of the labelling of the ATP plus ADP fraction of this tissue during the first 30 min. of incubation and completely inhibited further incorporation in the next 90 min. The ability of decanoate to alter the rate of incorporation of ^{32}P into slices of rat-brain cortex thus increases with the time of incubation, which suggests that a limited rate of penetration of the slice by the fatty acid may be involved.

In isolated rat-brain mitochondria the concentration of decanoate required to uncouple oxidation from phosphorylation by 50% is 0·6 mM (Scholefield, 1956). The sensitivity of rat-brain slices to a lower concentration of decanoate (0·3 mM), even in the first 30 min. of incubation, suggests that an effect of the membrane of the brain cells may be involved. Alternatively, the ATP–$^{32}P_i$ exchange reaction may account for much of the labelling of ATP with ^{32}P in slices, as has previously been shown to occur in liver (Ahmed & Scholefield, 1960). The results shown in Table 1 indicate that the exchange reaction in brain mitochondria is about as sensitive as is the process of oxidative phosphorylation, so that there is no reason to suppose that an exchange reaction is primarily responsible for the labelling with ^{32}P in brain slices. Indeed, the slow decline in the rate of labelling of the ATP in a cortex slice over the incubation period of 2 hr. and the failure to attain a steady-state level in this time would indicate otherwise. In liver, where the exchange reaction does take place rapidly in the mitochondria, the ATP of slices acquires a steady-state level of radioactivity within 20 min. (Ahmed & Scholefield, 1961). On the other hand, the observed rate of labelling in rat-brain-cortex slices in the absence of added inhibitor is much slower than would have been expected. Assuming a Q_{O_2} of 10 and a P/O ratio of 2·5 there should be approximately 45 μmoles of P_i incorporated into ATP/hr./100 mg. wet wt. of brain, and the observed average rate for the first 30 min. of incubation, based on incorporation of ^{32}P, is 45 μm-moles/hr. or 0·1% of the theoretical rate. It seems therefore that some of the membranes restricting the interchange of phosphate between the inorganic form and the ATP may well be intracellular.

Difficulties were at first encountered in assaying the ATP–$^{32}P_i$ exchange reaction in brain mitochondria. Media which support oxidative phosphorylation in liver also support the exchange reaction (Cooper & Lehninger, 1957). This is not so in brain, where the medium used to measure P/O ratios at 20° or 37° permitted only slow incorporation of ^{32}P into ATP by the exchange reaction at 20°. In the presence of protein (0·67% of bovine serum albumin) better exchange could be detected but the best results were obtained on addition of 10 mM-EDTA. A variable increase could also be detected on omission of Mg^{2+} ions from the incubation medium, but the most consistent results were obtained when both 10 mM-EDTA and 5 mM-Mg^{2+} ions were present. EDTA, besides removing factors which may be inhibitory to the exchange reaction, may also be chelating a considerable quantity of Mg^{2+} ions and maintaining a very low but critical concentration of these ions. This would account for the inhibition of the exchange reaction in brain by excess of Mg^{2+} ions in the presence of 10 mM-EDTA (Fig. 6).

Another factor leading to the conclusion that the labelling of the organic phosphates of brain slices with ^{32}P is due to net synthesis is the specificity of the substrate supporting the respiration. Of the substrates examined, only glucose brought about a high rate of phosphate incorporation, a rate of approximately six times the value observed in the absence of added substrate being obtained. Pyruvate and α-oxoglutarate slightly increased the phosphate incorporation whereas succinate and glutamate failed to raise the level above that of the endogenous, although the respiratory activity of the slices was high. Earlier studies on phosphate incorporation into brain slices showed that glutamate and succinate, though increasing the oxygen consumption of the slices, failed to support the incorporation of [^{32}P]phosphate into phospholipids, phosphoproteins, nucleic acids and residue organic phosphorus fractions (Findlay *et al.* 1953; Strickland, 1954). It is now shown that in the presence of these substrates the incorporation of [^{32}P]phosphate into ATP is as low as it is in the other fractions. Thus the low rate of incorporation of ^{32}P into the

other organic phosphates is probably a consequence of a low ^{32}P turnover in the ATP plus ADP fraction.

Weil-Malherbe (1950) has pointed out that glutamate may not be a major energy-yielding substrate in brain. A number of observations support this conclusion (see, for example, McIlwain, 1951*a*, *b*, 1953; McIlwain & Gore, 1953; Waelsch, 1951). Similar considerations may apply to succinate since Kratzing (1953) has shown that guinea-pig-brain slices respiring in succinate did not respond to electrical stimulations and also failed to maintain their levels of phosphocreatine and ATP. Quastel, Tennenbaum & Wheatley (1936) had already shown that optimum synthesis of acetylcholine in brain slices takes place only in the presence of glucose and oxygen. The synthesis was not supported by succinate and only partially supported by pyruvate or glutamate. Similarly, Stern, Eggleston, Hems & Krebs (1949), who studied the accumulation of glutamate in slices against a concentration gradient, found that of the various additional substrates studied only glucose supported the maximum rate of accumulation of glutamate. On the other hand, Abood & Gerard (1952) and Brody & Bain (1952) have shown that brain mitochondria may yield P/O ratios approaching the theroretical values during the oxidation of succinate, glutamate or other substrates. Similarly, labelling of phospholipids with ^{32}P in rat-brain mitochondria has been demonstrated in the presence of these substrates (McMurray, Berry & Rossiter, 1957). The oxidation of substrates such as succinate and glutamate by slices may not be properly coupled to phosphorylation or the ATP so formed may not be available for the synthetic processes or to the cellular pool of radioactive phosphate.

The rate of incorporation of ^{32}P into brain-cortex slices is much lower under anaerobic conditions than it is under aerobic conditions (Findlay *et al.* 1953; Strickland, 1954). In the present studies, incorporation of ^{32}P into the ATP plus ADP fraction under anaerobic conditions was about 5% of that observed under aerobic conditions. Low incorporation of ^{32}P into the ATP plus ADP fraction under anaerobic conditions might have been expected. The relative yields of ATP may be expressed (Quastel & Bickis, 1959) by the ratio $Q^{N_2}_{CO_2}/6 \times Q_{O_2}$. The observed values for $Q^{N_2}_{CO_2}$ and Q_{O_2} were 7 and 11 respectively, so that the ratio is 7/66 and the yield of ATP under anaerobic conditions would be expected to be about 10% of that to be obtained under aerobic conditions. This is of the same order as the figure of 5% quoted above for the ratio of the rates of labelling of ATP with ^{32}P and is in contrast with results from tumours, where it has been shown (Quastel & Bickis, 1959) that the Q_{ATP} in the presence of glucose is independent of the presence of oxygen.

SUMMARY

1. Decanoate and other fatty acids initially stimulated the respiratory activity of rat-brain-cortex slices incubated in Krebs–Ringer glucose medium. Subsequently the respiratory activity ceased gradually and irreversibly. The time interval between these two effects depended upon the concentration and chain length of the acid.

2. Of the fatty acids from heptanoate to myristate, heptanoate was the least effective as an inhibitor and decanoate was the most effective. Saturated fatty acids of greater chain length were less effective. Decanoate (0·8 mM) brought about an inhibition of respiration of rat-brain-cortex slices after an incubation period of 45–60 min.

3. The greatest inhibitory effects of decanoate were obtained when glucose was present as the substrate. When the acids of the carboxylic acid cycle were used as substrates the respiration was not inhibited to the same extent as with glucose, nor was the inhibition complete.

4. Decanoate and dinitrophenol greatly stimulated aerobic glycolysis at concentrations which were inhibitory to respiration. Anaerobic glycolysis was stimulated slightly by these agents.

5. Decanoate inhibited phosphate incorporation into all the organic phosphate compounds at concentrations which did not significantly alter the respiratory activity of rat-brain-cortex slices.

6. The adenosine triphosphate–$^{32}P_i$ exchange reaction in rat-brain mitochondria is not as rapid as it is in liver mitochondria. It is best measured in the presence of 10 mM-ethylenediaminetetra-acetate and at least 3 mM-Mg^{2+} ions. The inhibitory effects of decanoate on this exchange reaction and on coupled phosphorylation are quantitatively similar.

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Further Studies on the Absorption of Vitamin A

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Irrespective of whether vitamin A is fed to rats as the free alcohol or as its esters, 75 % of it is found in the esterified form in the mucosae and muscles of the small intestine, whereas only the ester is transported exclusively through the lymphatic system, so that in the post-absorptive blood the ester shows a sharp increase (Ganguly, 1960). Gray & Cawley (1942) concluded from the molecular-distillation data on the oil obtained from rat liver that vitamin A was probably stored in the liver as its palmitate. Later work of Kaiser & Kagan (1956) showed that the vitamin A ester of blood and liver of rats was composed of higher fatty acids, and Mahadevan, Krishnamurthy & Ganguly (1959) showed that the lower esters of vitamin A were not absorbed from the intestine.

Gray, Hickman & Brown (1940) suggested that when rats are fed with different types of esters of vitamin A the liver, in time, converts them into one type of ester, probably the palmitate. We have recently demonstrated that an enzyme preparation from the intestinal mucosae of rats can rapidly esterify vitamin A with almost equal efficiency with saturated or unsaturated fatty acids containing more than 10 carbon atoms. However, it fails to esterify the vitamin with acids containing fewer carbon atoms (Murthy, Mahadevan, Seshadri Sastry & Ganguly, 1961). It might therefore be assumed that different types of esters (depending upon the availability of fatty acids of either endogenous or exogenous source) would be present in the mucosae and muscles of the small intestine, as well as in the blood and liver of rats, after a dose of the vitamin. It is demonstrated here that contrary to this expectation, it is the palmitate of vitamin A that always predominates in the mucosae, muscles and blood, and only the palmitate is deposited in the liver of rats.

MATERIALS AND METHODS

Methanol (Merck; Pro Analyst grade) and butanol (British Drug Houses Ltd.; Laboratory Reagent grade) were redistilled before use. Tweens 20, 40 and 60 (lauric, palmitic and stearic acid esters respectively of polyoxyethylene sorbitol) were obtained from Atlas Powder Co., Wilmington, Del., U.S.A. Refined coconut oil was obtained from the local market. Groundnut, sesame (*Sesamum indicum*) and safflower (*Carthamus tinctorius*) oils were expressed in an expeller from the respective oil seeds commercially available and were used without further purification. Silicone fluid F 110/300 was from Imperial Chemical Industries Ltd., London. The rest of the materials have been described in previous papers (Mahadevan *et al.* 1959; Ganguly, Krishnamurthy & Mahadevan, 1959).

Synthetic vitamin A alcohol, purified by chromatography on alumina columns (Ganguly, Krinsky, Mehl & Deuel, 1952), was dissolved in light petroleum (b.p. 40–60°) together with the Tween or the oil so as to obtain a final concentration of 6 mg. of the vitamin/ml. of the carrier. The solvent was evaporated under vacuum at 40–50°, the last traces being removed with a gentle stream of N_2. Colloidal dispersions were prepared according to Mahadevan *et al.* (1959).

Rats of either sex of this Institute strain were maintained after weaning on a vitamin-A-low diet composed of (%): starch, 65; solvent-extracted casein, 20; groundnut oil, 10; salt mixture, 5; with daily supplements of adequate amounts of the other vitamins. The animals were used after they had been on the diet for 5–6 weeks; at this stage their livers were essentially free from vitamin A.

After starvation for 24 hr. the animals were given an oral dose of 0·5 ml. of the carrier containing 3 mg. of vitamin A alcohol and were killed 2–3 hr. later by drawing as much blood as possible from the heart, while under light ether anaesthesia. Separation of the contents, mucosae and muscles of the small intestine, and extraction of these and of blood and liver were carried out as described earlier (Ganguly *et al.* 1959; Mahadevan *et al.* 1959). The extracts were chromatographed through alumina columns and the ester fractions were collected (Ganguly *et al.* 1952) for further analysis. Since the esterified vitamin A alone is transported from the intestine, this work was concerned with the study of the esters only; the alcohol fraction was not taken into account.

Separation of the higher fatty acid esters of vitamin A by reverse-phase circular chromatography on silicone-impregnated papers

Paper-chromatographic techniques were used for the separation of vitamin A alcohol, acetate and palmitate (Brown, 1953), and of the acetate and the higher esters (Kaiser & Kagan, 1956; Mahadevan *et al.* 1959). These techniques cannot resolve the higher esters of vitamin A, whereas our present work required a successful resolution of these esters. We obtained the best separation in a system where the filter paper is impregnated with silicone fluid and is irrigated with a mixture of methanol–butanol–water (85:10:5, by vol.).

The esters used as reference materials were prepared enzymically. A water extract (1 ml.) of 100 mg. of acetone-dried powder of rat pancreas and 1 ml. of ethanol containing 2 mg. of pure vitamin A alcohol plus 6 mg. of the required fatty acid were incubated at 37° for 1 hr. in 8 ml. of 0·01 M-phosphate buffer (Na_2HPO_4–NaH_2PO_4) at pH 6·6. At the end of the incubation the vitamin A was twice extracted with light petroleum after the addition of 10 ml. of ethanol. The esters formed were then separated from the unchanged vitamin A alcohol by chromatography on alumina columns (Ganguly *et al.* 1952).

Whatman no. 3 or no. 1 filter paper (45 cm. × 45 cm.) was dipped into a 7·5% (v/v) solution of silicone fluid in light petroleum for 30 sec. and allowed to dry at room temperature for 24 hr. The impregnated paper keeps for a considerable length of time without any change in its properties.

Usually the circular chromatogram was developed in the dark in large wooden chambers in an atmosphere of N_2 after spotting 10–15 μl. of light petroleum solutions containing 5–10 μg. of the ester at each point on the circumference of a circle drawn in the centre of the paper. Use of methanol alone for irrigation gave satisfactory separation, but addition of butanol cut down the length of the run, and the presence of water gave very sharp bands. With the solvent system of methanol–butanol–water (85:10:5) the separation was usually complete within 10–12 hr. The chromatogram was inspected under ultraviolet light and the fluorescent bands were marked. The rest of the procedure for the quantitative estimation of the esters was as described before (Mahadevan *et al.* 1959). Mixtures of known esters were chromatographed together with the extracts. It was extremely important to keep the chromatograms in the dark and not expose them to ultraviolet light while the irrigation was going on, as this led to the appearance of several additional fluorescent bands.

The recovery of the esters with this procedure was 90–95% and the R_F values were as follows: stearate, 0·29; palmitate, 0·32; myristate, 0·36; laurate, 0·4; oleate, 0·32; linoleate, 0·36; linolenate, 0·4. Complete separation of the saturated acid esters or of the unsaturated esters was possible but the 'critical pairs' (e.g. palmitate and oleate etc.) could not be separated.

For convenience, throughout this paper these bands will be referred to as if the saturated acids alone were present (e.g. stearate, palmitate, myristate and laurate). It is quite possible that the bands, especially those from the contents and mucosae may contain considerable amounts of the unsaturated esters of identical R_F values. This does not affect the objects and conclusions of this work to any extent, because we are demonstrating here that only one of these critical pairs, the 'palmitate', is actively transported and deposited. Also, as will be seen later on, we have been able to prove that this fraction of the intestinal muscles, blood and liver is composed almost entirely, if not wholly, of the palmitic acid ester.

RESULTS

Effect of carriers on the fatty acid composition of the vitamin A esters in the rat

Representative results of experiments, where different types of carriers were used, are summarized in Table 1. It is most striking that in the absence of exogenous palmitic or oleic acid an almost constant amount of the 'palmitate' was always present in the contents of the small intestine, as will be seen from the experiments with colloidal vitamin A alcohol, and with Tween 20 and Tween 60 dispersions of vitamin A alcohol. This same amount of the 'palmitate' was probably common in all other cases also, so that their enhanced 'palmitate' values are obviously due to the palmitic or oleic acid contributed by the carrier. The increase in the other esters is clearly due to the exogenous fatty acids. Thus, with Tween 20 (a laurate) and Tween 60 (a stearate), the 'laurate' and 'stearate' esters respectively rose sharply, whereas Tween 40 (a palmitate) gave rise to an extra amount of the 'palmitate'. Coconut oil, containing about 48% of lauric acid, yielded considerable quantities of vitamin A 'laurate', and, since this particular oil contains practically no linolenic acid, the ester should represent the lauric acid ester. The 'myristate' band was prominent with the other three oils, which have practically no myristic acid but are rich in linoleic acid, so that the materials of this band should be mostly vitamin A linoleate. It is thus obvious that the patterns of the vitamin A esters of the intestinal contents reflect the fatty acid composition of the vehicle used.

In the mucosae the ester composition in every case was very similar to that of the contents with the exception that the 'palmitate', as compared with the other esters, was more prominent. Where colloidal vitamin A was given, the 'palmitate' con-

centration was very high, and was practically the only ester.

The picture of the esters in the intestinal muscles was radically different from that in the mucosae. Whereas the esters of the mucosae largely reflected the fatty acid composition of the vehicle, only the concentration of the 'palmitate' was of any real significance in the muscles, because in all cases it was present to the extent of 79–93 % of the total esters. However, smaller quantities of the other esters representing the fatty acid composition of the carrier persisted. Thus when Tween 20 and coconut oil were used, there were small amounts of the 'laurate', whereas Tween 60 gave some 'stearate' and with the other three oils some of the 'myristate' was present. The ester composition of the blood appears to resemble that of the intestinal muscles: the 'palmitate' predominated in all the samples.

The liver, on the other hand, presents a rather unique picture since only the 'palmitate' was stored in spite of the presence of other esters in the blood.

Identification of the fatty acid of the vitamin A ester of intestinal muscles, blood and liver

These results show that only one of the critical pairs, i.e. the 'palmitate', is admitted inside the animal body to any significant extent. But, as pointed out earlier, the chromatographic procedure adopted here cannot resolve the critical pairs. Attempts were therefore made to obtain more definite information about the nature of the fatty acid of the 'palmitate' band of the muscles, blood and liver. These tissues from 10 rats, dosed with similar amounts of vitamin A alcohol dissolved in groundnut oil and killed 2–3 hr. later, were extracted according to Ganguly *et al.* (1959). The tissue extracts in light petroleum were shaken in separatory funnels five or six times with equal volumes of fresh aq. 85 % (v/v) ethanol each time. The ethanol layers were discarded: the light petroleum layers were twice chromatographed on alumina columns when the ester fractions were eluted with 1 % (v/v) of acetone in light petroleum. The esters were now chromatographed in batches on silicone-impregnated filter papers; the other smaller bands were discarded and only the 'palmitate' band was recovered. The paper-chromatographic procedure was repeated once more, after which the eluate was finally passed through an alumina column. The esters, thus purified, were saponified by heating with alcoholic potassium hydroxide (20 %, w/v) on a water bath at 60–80°; the fatty acids were then extracted with light petroleum after acidification with hydrochloric acid.

Duplicate samples of the liberated fatty acids were now separately subjected to reverse-phase chromatography on paraffin-impregnated papers with two different solvent systems. One of the chromatograms was developed with 90 % acetic acid and the other with acetic acid–formic acid–20 % hydrogen peroxide (6:1:1, by vol.). The latter system is known to destroy all the unsaturated acids and to carry the products to the solvent front

Table 1. *Vitamin A ester composition of the contents, mucosae and muscles of the small intestine, and of the blood and liver, of rats 2 to 3 hr. after feeding with vitamin A alcohol in different carriers*

Each rat was given orally 3·0 mg. of vitamin A alcohol in 0·5 ml. of the Tween or oil or in 0·5 ml. of water containing 1 % of sodium taurocholate. The results are averages of three separate experiments with individual rats. Values are expressed as μg. of vitamin A alcohol present as ester per whole organ in contents, mucosae, muscles and liver, and per 10 ml. of blood. S, 'Stearate'; P, 'palmitate'; M, 'myristate'; L, 'laurate'.

Carrier used	Main fatty acid(s) present	Intestine: Contents S	P	M	L	Mucosae S	P	M	L	Muscles S	P	M	L	Blood S	P	M	L	Liver S	P	M	L
Colloidal dispersion in water	None	8	27	0	0	6	143	2	4	2	78	1	1	0	30	0	0	0	153	0	0
Tween 20	Lauric	11	26	3	70	15	107	19	67	3	133	3	12	2	39	2	8	0	116	0	0
Tween 40	Palmitic	14	92	0	0	19	219	9	13	2	207	3	5	0	45	0	0	0	179	0	0
Tween 60	Stearic	62	23	3	0	53	99	8	7	14	115	1	1	3	37	0	0	0	115	0	0
Coconut oil	Lauric	10	48	12	48	28	104	12	86	6	126	2	17	1	29	1	3	0	136	0	0
Groundnut oil	Oleic, linoleic	4	60	30	7	23	126	58	9	5	128	15	2	1	28	3	1	0	159	0	0
Sesame oil	Oleic, linoleic	2	47	43	0	9	117	93	7	3	135	22	2	2	23	6	1	0	145	0	0
Safflower oil	Linoleic, oleic	2	42	52	3	6	113	67	3	2	83	19	1	1	18	4	1	0	127	0	0

leaving the saturated ones unaffected (Buchanan, 1959). The positions of the fatty acids were located by treating the papers with cupric acetate solution followed by potassium ferrocyanide (Wagner, Abisch & Bernhard, 1955). In both chromatograms of samples from intestinal muscles, blood and liver only one band, corresponding to palmitic acid, was present, and by visual comparison no difference in the intensity of the colour between the peroxide-treated and untreated chromatograms could be observed.

Since the liver contained larger amounts of the ester, we were able to use other methods to prove that it is a palmitate. Representative samples of the liver ester were purified as described above. From a portion of the purified ester fraction the exact amount of vitamin A present was accurately determined, expressed as vitamin A alcohol (1·36 mg.). From this vitamin A alcohol value, the theoretical amount of palmitic acid, that should combine with it, was calculated (1·22 mg.). The rest of the sample was saponified and the fatty acids were chromatographed on paper with and without H_2O_2 as described above. The paper was treated with 0·1% mercuric acetate solution, washed thoroughly with running tap water, dried and sprayed with a solution of 0·2% diphenylcarbazide reagent. The coloured bands thus formed were eluted with toluene and methanol, and the quantity of fatty acids present was determined colorimetrically (Viswanathan & Meera Bai, 1961). There was no difference in the values between the peroxide-treated and untreated samples. Also, the amount of palmitic acid estimated (1·27 mg.) agreed closely with the theoretical value as calculated above. Finally, the iodine vapour test of another chromatogram was negative. Thus although oleic acid is more abundant than palmitic acid in groundnut oil, there was practically no vitamin A oleate in the intestinal muscles, blood or liver of rats.

Changes in the ester composition of vitamin A in the rat with time

Gray *et al.* (1940) indicated that several esters of vitamin A might reach the liver to be eventually converted into one type, probably the palmitate. The above experiments have demonstrated that, irrespective of the ester composition of the contents and mucosae, the liver can store only the palmitate. These experiments, however, were carried out within a very short time interval (2–3 hr.) after dosing and hence it remained a possibility that given longer time intervals the liver might store the other esters in addition to the palmitate. Rats were therefore fed with vitamin A alcohol in coconut oil and killed at the stated time intervals. The relative appearances of the different esters with respect to time in the various tissues of these rats are tabulated in Table 2. Up to 6 hr. the ester patterns in all the samples were similar to those obtained with the same oil in the experiments of Table 1. However, after 10 hr. the palmitate became the most prominent constituent, and after 24 hr. it was the sole component. Here again, despite the fact that lauric acid is the major acid of coconut oil, at no time did the liver show any traces of any other ester but the palmitate, even though the total ester stored per liver increased from 125 μg. at 2 hr. to 1200 μg. after 24 hr.

Fatty acid specificity for the esterification of vitamin A by the enzymes of the intestinal muscles

The ester patterns in the contents and mucosae are probably due to the rather non-specific nature of esterification of vitamin A by the enzymes of the pancreas and mucosae. The predominance of the palmitate in the intestinal muscles, on the other hand, might well be due to a specific esterification of vitamin A brought about by the muscle enzyme with palmitic acid. The esterifying activity of the acetone-dried powder of the muscles with different

Table 2. *Vitamin A ester composition of the contents, mucosae and muscles of the small intestine, and of the blood and liver of rats, at different time intervals after feeding with vitamin A alcohol in coconut oil*

Each rat was given orally 3·0 mg. of vitamin A alcohol in 0·5 ml. of coconut oil. The results are averages of three separate experiments with individual rats. Values are expressed as μg. of vitamin A alcohol present as ester per whole organ in contents, mucosae, muscles and liver, and per 10 ml. of blood. S, 'Stearate'; P, 'palmitate'; M, 'myristate'; L, 'laurate'.

	Time after dosing																							
	2 hr.				3 hr.				4 hr.				6 hr.				10 hr.				24 hr.			
Tissue	S	P	M	L	S	P	M	L	S	P	M	L	S	P	M	L	S	P	M	L	S	P	M	L
Intestine																								
Contents	9	37	8	56	8	53	7	58	4	42	3	37	4	39	4	26	5	23	3	15	2	12	0	0
Mucosae	6	59	6	38	14	128	5	92	13	132	5	89	10	78	4	47	1	39	1	5	0	5	0	0
Muscles	4	67	4	18	6	132	5	15	6	138	4	19	4	84	3	13	0	48	1	4	0	8	0	0
Blood	1	14	1	3	1	23	1	4	1	32	1	8	1	27	1	5	0	18	0	2	0	9	0	0
Liver	0	125	0	0	0	170	0	0	0	376	0	0	0	729	0	0	0	820	0	0	0	1200	0	0

fatty acids was therefore tested as described by Murthy *et al.* (1961). The relative esterification with the different acids, as shown in Table 3, was similar to that obtained with the mucosal and pancreatic enzymes, inasmuch as no preference for any particular acid was exhibited. In a separate experiment, where an ethanolic solution of 1 mg. of vitamin A alcohol was similarly incubated with a mixture containing 1 mg. each of lauric, myristic, palmitic and stearic acids, no significant preference for esterification with palmitic acid was noticed.

It is also possible that blood and liver might bring about the formation of larger amounts of the palmitate by means of a mechanism of transesterification with other lipid materials, without going through the processes of hydrolysis and re-esterification [these two latter processes have been shown to be absent from these tissues (Mahadevan, Murthy, Krishnamurthy & Ganguly, 1961)]. Experiments were therefore carried out in which 0·5 ml. of an ethanolic solution of the esters (laurate, myristate, palmitate and stearate) containing 250 μg. of each was separately incubated in 4 ml. of phosphate buffer (pH 6·6; 0·1 M) with 1 mg. of Tween 40 and 1 ml. of rat-liver homogenate (equivalent to 500 mg. of wet liver) or 1 ml. of plasma for 1 hr. at 37°. No formation of any additional palmitate was observed.

Nature of vitamin A esters in the livers of other species

Samples of vitamin A ester fractions from the livers of chickens, sheep, shark and rats, when chromatographed in our system, revealed that shark and rat liver display only one band, corresponding to the 'palmitate', whereas the chicken-liver sample resolved itself into two distinct bands representing about 70 % of 'palmitate' and 30 % of 'stearate'. In sheep liver the 'palmitate' was prominent, accounting for about 90 % of the total esters.

Table 3. *Fatty acid specificity for the esterification of vitamin A by the enzymes of rat intestinal muscles*

Conditions and procedures were as described by Murthy, Mahadevan, Seshadri Sastry & Ganguly (1961) except that the enzyme extract added contained 20 mg. of protein.

Fatty acid	Vitamin A alcohol esterified (μg.)
Lauric	64·2
Myristic	54·9
Palmitic	53·7
Stearic	51·4
Oleic	57·5
Linoleic	58·3
Linolenic	54·6

DISCUSSION

The techniques employed in the earlier work of Kaiser & Kagan (1956) and of Mahadevan *et al.* (1959) were not suitable for the resolution of the higher esters of vitamin A, so that it was possible to arrive only at the broad conclusion that the higher esters are admitted into the rat. With the successful separation of these esters (or pairs of esters), we have been able to conclude in the present report that this ester in the rat is a palmitate. In no case have we been able to detect in the tissues of the rat vitamin A esters with acids shorter than lauric acid, fully confirming the earlier findings of Kaiser & Kagan (1956), Mahadevan *et al.* (1959) and of Bruggemann & Tiews (1960). However, Loran & Althausen (1959) have claimed that in an isolated intestine *in vitro* the long-chain esters of vitamin A are hydrolysed on the lumen side and are re-esterified inside the mucosae with acetic acid. This is in sharp disagreement with the information given in our earlier experiments (Mahadevan *et al.* 1959) and in the present paper. We have already demonstrated that the intestinal enzymes preferentially esterify vitamin A with long-chain acids, whereas no esterification could be obtained with acetic acid. On the other hand, as compared with the higher esters, vitamin A acetate is more rapidly hydrolysed by the enzymes of this tissue (Mahadevan *et al.* 1961).

The fatty acid composition of the carrier employed for vitamin A is reflected by the patterns of the esters in the lumen and mucosae of the small intestine of rats. This can readily be explained, because the pancreas and mucosae of rats esterify vitamin A non-specifically with long-chain acids. But 'palmitate' is almost the sole constituent in the absence of exogenous fatty acids, e.g. after feeding with colloidal vitamin A. It appears preferentially in the intestinal muscles, in spite of the presence of considerable amounts of other esters in the mucosae, as found when Tween 20 or 60 or one of the four oils is used as the carrier. This cannot be explained on the basis of the assumption of any preferential esterification of the vitamin in this tissue with palmitic acid alone, because the muscle enzyme, like the mucosal enzyme, seems to be rather non-specific towards the fatty acid for this process. Nor can this be explained by assuming ready availability of palmitic acid in the intestine, because according to Coniglio & Cate (1958) this acid is not the only major fatty acid in the normal intestinal tissue of rats. The failure to demonstrate any transesterification mechanism in the blood and liver rules out the possibility of such reactions accounting for the presence of 'palmitate' only in these tissues.

One possible explanation seems to be that a certain amount of specificity for the vitamin A

palmitate exists in the mechanism of its transport from the mucosae onwards and in its final storage in the liver. Indeed Krinsky, Cornwell & Oncley (1958) have demonstrated the specificity of lipoproteins in their binding with vitamin A ester and vitamin A alcohol in post-absorptive human blood. In an earlier paper we also have discussed and suggested a similar possibility in the transport and storage of vitamin A in the rat (Ganguly *et al.* 1959), and this aspect has been more exhaustively reviewed by Ganguly (1960). Krinsky *et al.* (1958) are of the view that the specific lipoprotein, responsible for the transport of vitamin A ester in blood, occurs in the Kupffer cells of the liver. It is possible that this lipoprotein exists in the Kupffer cells, in blood and in the central lacteals of the intestinal villi. The 'palmitate', even in the mucosae, could then be in actual combination with this lipoprotein and probably inside the central lacteals. The picture in the intestinal muscles would then appear to represent that phase of absorption where the ester, after entering the lymphatic system in the lipoprotein-bound state, has travelled far away from the actual site of esterification (the mucosae) on its way to the systemic blood, so that mostly the palmitate is found. Since the Kupffer cells of the liver contain the same lipoprotein, it preferentially removes only the palmitate from the systemic blood and totally rejects the others.

McGillivery, Thompson & Worker (1957) have demonstrated that, on intravenous injection of emulsions of vitamin A alcohol or palmitate in rats and goats, the palmitate is more efficiently taken up by the liver, whereas more of the administered alcohol keeps on circulating in the blood. Preliminary experiments being carried out by us also indicate that, after intravenous injection of vitamin A stearate dispersed in water with Tween 40, no stearate could be detected in rat liver. These observations would lend further support to the hypothesis that rat liver selects only the palmitic acid ester of vitamin A.

There were, however, traces of the other esters in the intestinal muscles and blood, but not in the liver. Since the liver has no acceptors for them, they tend to accumulate in the circulating blood either to be detoxicated or to be hydrolysed and re-esterified with palmitic acid in the intestine or pancreas during their rapid circulation, so as to be deposited ultimately in the liver as the palmitate.

To summarize, it would thus appear possible that the lipoprotein exerts its selective binding probably at the mucosal cells for the palmitate only, the bound palmitate then travels through the lymphatic system and finally finds its way to the Kupffer cells, where it is deposited and the free lipoprotein comes back to the intestine to carry more palmitate.

SUMMARY

1. A circular paper-chromatographic procedure for the separation of higher fatty acid esters of vitamin A, with silicone-impregnated paper and a solvent system of methanol–butanol–water (85:10:5, by vol.), is described.

2. Rats raised on a vitamin-A-low diet and starved for 24 hr. were dosed with vitamin A alcohol in the carriers Tweens 20, 40 and 60, groundnut oil, sesame oil, coconut oil or safflower oil; the vitamin A ester fractions of the contents, mucosae and muscles of the small intestine, and of blood and liver, were analysed by the above method 2–3 hr. after the dose.

3. After the feeding of colloidal vitamin A, mostly vitamin A palmitate was found in the contents, mucosae and muscles of the small intestine, and in the blood and liver, of rats.

4. The types of the esters of the contents and mucosae in all other cases were governed by the fatty acids of the carrier. No such relationship was found for the ester composition of intestinal muscles and blood, which contained almost entirely the palmitic acid ester. The liver, under all conditions studied, stored exclusively the palmitate.

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Cellular Components of *Bacillus megaterium* and their Role in Protein Biosynthesis

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Previous reports from this Laboratory (Butler, Crathorn & Hunter, 1958; Hunter, Brookes, Crathorn & Butler, 1959) have described the preparation of a membrane complex ('cytoplasmic membrane fraction') from protoplasts of *Bacillus megaterium*. When prepared under defined conditions, the membrane complex contained the complete apparatus necessary for the incorporation of labelled amino acids into polypeptide bonds. Intermediate reactions in the process were also studied and it was shown that the membrane complex contained enzymes, capable of activating amino acids, and also RNA that binds amino acids into bonds labile to the action of dilute alkali and ribonuclease. In the present work, the nature of the membrane complex has been examined in greater detail, and it has been possible to separate several components by mild physical methods. These components, which include ribonucleoprotein particles and a phospholipoprotein fraction, have been analysed by both physical and chemical methods, and an attempt has been made to assess their individual contributions to the protein-synthetic mechanism.

METHODS

Organism. The strain of *B. megaterium* used (originally KM) was cultured as described by Butler *et al.* (1958). For all the experiments described here the organism was grown at 30° in a glucose–salts medium (C medium; McQuillen, 1955).

Preparation of the membrane complex and cytoplasmic fraction. Protoplasts were prepared by gentle shaking at 30° in the presence of 0·02% lysozyme and in a medium of the following composition (%, w/v), finally brought to pH 7·0 with NaOH: glucose, 1; NH_4Cl, 0·2; Na_2HPO_4, 0·6; KH_2PO_4, 7·15; NaCl, 0·3; $Na_2SO_4,10H_2O$, 0·025; $MgCl_2,6H_2O$, 0·222 (this will be termed below as 0·5M-phosphate–salts medium). Cells from early logarithmic-phase cultures were usually completely converted into protoplasts within 30 min. When microscopic examination failed to reveal the presence of a single whole cell in a sample, the protoplasts were harvested and then osmotically lysed in one of two ways.

In the procedure designated 'conc. lysis' the protoplast pellet (1–2 g.) was resuspended in 1 vol. (80 mg./ml.) of 0·5M-phosphate–salts medium, diluted with 1·5 vol. of water and vigorously shaken by hand until no whole protoplasts could be seen under the microscope. When the weight of protoplasts was smaller (0·2–0·5 g.), the pellet was immediately suspended in the diluted medium and shaken as before. The resulting suspension was centrifuged at 105 000*g* for 10 min. to sediment the membrane complex, the supernatant being termed the cytoplasmic fraction.

In the procedure designated 'dil. lysis', the protoplasts were lysed as above, but in this case at least 15 vol. of water was used. Lysis under these conditions is very rapid and the membrane complex and cytoplasmic fractions were collected as before. In experiments where it is undesirable to use phosphate media (e.g. experiments on pyrophosphate exchange), tris (brought to pH 7·2 with HCl) was used at final concentration of 0·6M for 'conc. lysis' and 0·01M for 'dil. lysis'.

Fractionation of the membrane complex. The membrane complex was disintegrated in buffered salt media by a variety of methods. The normal procedure was to treat the membrane complex (approx. 50 mg./ml.) with ultrasonic vibrations (from an M.S.E.–Mullard machine) for 3 min. The operation was performed in a plastic tube (12 ml.) cooled in an ice bath. In early experiments, a Mickle glass-bead tissue disintegrator was used, but it was subsequently found that the same result could be obtained simply by shaking the membrane complex vigorously by hand until it had been visibly dispersed. Provided the membrane complex is completely dispersed with no large lumps visible, the method and the volume of solution are unimportant. Ultrasonic disintegration was preferred in these experiments as the volume can be kept small and the operation can be carried out rapidly. The treatment was usually carried out in 0·03M-phosphate–salts medium or 0·01M-tris and the concentration of Mg brought to 0·01M.

The resulting suspension was centrifuged at 50 000*g* for 25 min. The supernatant fraction was removed, the tube cut, and the two layers of the sediment were separated with a spatula. The lower layer mucoprotein fraction was so hard packed that the upper layer (phospholipoprotein fraction) could be removed without contamination. If a clean uncontaminated sample of the mucoprotein fraction was required, it was resuspended and resedimented, and the fine film of the top layer removed.

The supernatant fraction removed from the two sedimented layers contained ribonucleoprotein particles (ribosomes, 30s and 45s) as well as free protein and RNA (approx. 5s). These components were separated in a discontinuous sucrose-density gradient. The sucrose solutions also contained 0·01M-$MgCl_2$ and 0·03M-KH_2PO_4 adjusted to pH 7·0 with NaOH. The density gradient was constructed by

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layering 8 ml. each of solutions containing 1·25 M-, 0·75 M- and 0·5 M-sucrose in a centrifuge tube about 1 hr. before use. All the operations were performed at 2–5°. The solution containing ribosomes (3–4 ml.) was layered on the top, and the gradient spun at 20 000 rev./min. in a Spinco SW. 25 head for more than 6 hr. The ribosomes sediment to a visible band about two-thirds of the way down the tube while the free protein and RNA barely pass the boundary into the 0·5 M-sucrose. The bottom of the tube was pierced with a hypodermic needle and the emergent drops were collected in a series of fractions. A sample of each fraction was diluted. The extinction coefficients at 260 mμ indicated the distribution of three distinct separated components, namely 30s, 45s and 5s material. Each fraction was normally precipitated directly with cold 30% trichloroacetic acid to a final concentration of 5%, and the precipitate recovered by centrifuging. If desired the ribosomes can be sedimented out of the sucrose media by centrifuging at 105 000*g* for 4 hr.

Experiments with the isolated membrane complex. When the uptake of [^{14}C]amino acids by the various fractions of the membrane complex was being studied, the membrane pellet was thoroughly shredded into small pieces with a spatula and then resuspended in 50 ml. (2 mg./ml.) of 0·5 M-phosphate–salts medium and equilibrated by shaking at 30° for 30 min. To terminate the incubation 0·2 mg./ml. of cold chloramphenicol was added and the incubation flask plunged immediately into an ice bath. Such treatment inhibits the incorporation of [^{14}C]amino acids within 2–3 sec. The fragments of the membrane complex are then spun out of the incubation mixture by centrifuging in the cold at 105 000*g* for 5 min. The labelled membrane complex is then separated into its components as indicated above. The presence of chloramphenicol at this stage is unimportant and does not affect the distribution of label among the fractions.

Experiments with protoplasts. In later experiments, protoplasts were employed because they normally incorporate [^{14}C]amino acids faster than the isolated membrane complex and the conditions of protein synthesis are nearer to those of a whole cell. The protoplasts were separated in 0·5 M-phosphate–salts medium (about 50 mg./ml.), equilibrated by shaking at 30° for 1 hr. and incubated with [^{14}C]amino acids, and the labelling was terminated as for the membrane complex. The protoplasts were harvested in the cold and lysed in concentrated buffer in the presence of chloramphenicol (0·2 mg./ml.), and the membrane complex obtained was fractionated as above. The label in the cytoplasmic fraction was also recorded in these experiments. The protoplasts are quite stable in 0·5 M-phosphate–salts medium and can be fairly vigorously shaken.

Safeguards against contamination with whole cells. B. megaterium is so large (5 μ) that whole cells can easily be seen under the high power of a phase-contrast microscope (×1000). Preparations of protoplasts were never used if a single whole cell could be found in a sample of the lysozyme digest. Similarly, preparations of the membrane complex were examined for whole cells. A further indication that no whole cells were present was the very small amounts of radioactivity incorporated into the mucoprotein fraction. Whole cells would have remained unbroken by the mild fractionation procedure and would have sedimented with the mucoprotein fraction.

Experiments on amino acid-activating enzymes. To test for amino acid-activating enzymes the cell fractions were prepared in phosphate–salts or tris media, as indicated in the text. The final incubation mixture contained 2 mM-KF, 2 mM-$MgCl_2$, 2 mM-ATP, a mixture of amino acids giving a final concentration of 4 mM, an enzyme preparation (about 80 μg. of protein/ml.) in a final volume of 2 ml. buffered to pH 7·2 with tris or phosphate–salts medium. Incubations were carried out at 37° for 30 min. in the presence of 0·25 μc of sodium [^{32}P]pyrophosphate, the reaction was terminated with 2 ml. of 10% trichloroacetic acid, and the ATP isolated by absorption on charcoal as described by Crane & Lipmann (1953). The washed charcoal was finally boiled for 10 min. with N-HCl and the radioactivity of the hydrolysate determined.

Assays of radioactivity. The protein samples were prepared for radioactive assay by the methods previously described (Butler *et al.* 1958; Crathorn & Hunter, 1957). Assay of solid samples was carried out by end-window counting as described by Butler *et al.* (1958).

^{32}P samples were counted in 10 ml. samples in an annular liquid counter Type M6M (20th Century Electronics Ltd., New Addington, Surrey).

Chemical analyses

Estimation of ribonucleic acid. RNA was estimated by the orcinol method (Ceriotti, 1955). The results were checked on another sample by measurement of the extinction at 260 mμ in a Unicam spectrophotometer (SP. 600) after extraction of the RNA by the Schmidt–Thannhauser procedure, and with an extinction coefficient of 32·4 mg./ml./cm. (Scott, Fraccastoro & Taft, 1956).

Estimation of protein. In all fractions except the bottom sediment, which contains a large amount of carbohydrate, the weight of the material insoluble in hot trichloroacetic acid after extraction of the lipids was taken as the weight of protein. This was checked by determining the protein according to Lowry, Rosebrough, Farr & Randall (1951), insoluble protein samples being dissolved in N-NaOH. The protein contents of the particulate fractions were checked by determining the N content by a micro-Kjeldahl method and multiplying by 6·5. Standard curves, which were closely similar, were prepared from lysozyme, ribonuclease or serum albumin, all dissolved in N-NaOH. All the final mixtures contained the same amount of alkali.

Estimation of lipid. The lipid was determined by the loss in weight of a sample after extraction by boiling for 30 min. in chloroform–ethanol (1:1, v/v). The extract was then evaporated to dryness and the residue weighed to check the first estimate. The inorganic phosphorus present in the extract was determined after destruction of all organic material with 60% (w/w) $HClO_4$ (A.R.) by the method of Fiske & Subbarow (1925). By using 3·5% as the phosphorus content of phospholipid (Weibull & Bergström, 1958) the phospholipid can be calculated. Some protein is solubilized by the chloroform–ethanol solvent so that the lipid content is slightly high by the weight difference method, but, as *B. megaterium* contains some neutral lipid (Hunter & Goodsall, 1961), this method may be more accurate than that of Weibull & Bergström (1958).

Electron microscopy. Samples were prepared and fixed by the methods described by Mercer (1959).

Sedimentation in the ultracentrifuge. The sedimentation velocity experiments were performed in a Spinco model E

ultracentrifuge at 35 500 rev./min., the observed sedimentation coefficients being corrected to water at 20° in the normal way.

Materials. Generally labelled L-[^{14}C]amino acids (5–9 μc/μmole) were supplied by The Radiochemical Centre, Amersham, Bucks., and were used as supplied without any dilution with [^{12}C]amino acids.

Sodium [^{32}P]pyrophosphate was also obtained from The Radiochemical Centre.

RESULTS

Components of the membrane complex. Protoplasts of *B. megaterium* can be lysed in two ways: either by shaking in a dilute buffer (0·03 M-phosphate–salts or 0·01 M-tris) when the lysis is instantaneous or by diluting a concentrated buffer medium just sufficiently to effect osmotic rupture (0·3 M-phosphate–salts or 0·6 M-tris). The two processes are termed below 'conc. lysis' and 'dil. lysis'. It has been found that the slow-speed sediment (membrane complex) obtained after conc. lysis of the protoplasts is capable of incorporating [^{14}C]amino acids into protein peptide bond, but the membrane complex obtained by dil. lysis is unable to do this. In each case about 50 % of the total cellular RNA is deposited with the membrane complex, and, if this complex is washed with a dilute buffer (0·03 M-phosphate–salts or 0·01 M-tris, each containing 0·01 M-$MgCl_2$ and adjusted to pH 7·2 with NaOH or HCl), 30s and 45s ribosomes are released together with amino acid-activating enzymes, free protein and RNA (Figs. 1 and 2; Table 2). The RNA analyses given in Table 1 show that approximately 57 % of the RNA present in the protoplasts is ribosomal and after dil. lysis about 67 % of these ribosomes are sedimented with the membrane complex. Approximately 70 % of the RNA bound to the membrane complex (both dil.

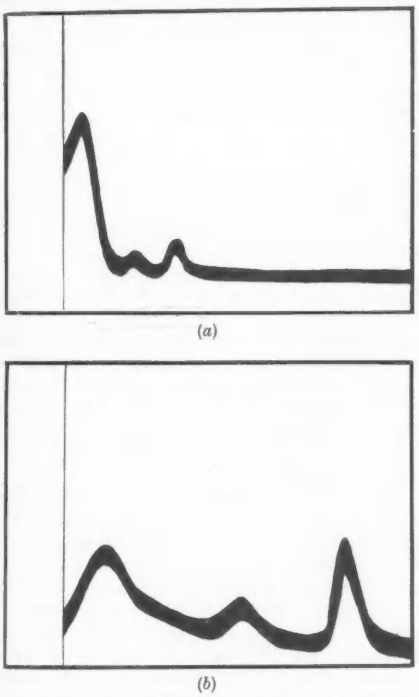

Fig. 1. Sedimentation diagrams of fractions obtained when protoplasts are lysed in 0·03 M-phosphate–salts medium. (*a*) Cytoplasmic fraction, photographed at 12 min. after attainment of full speed. (*b*) Material released from membrane complex when it is washed in 0·01 M-tris + 0·01 M-$MgCl_2$, pH 7·2, photographed at 28 min. after attainment of full speed. For full experimental details, see text.

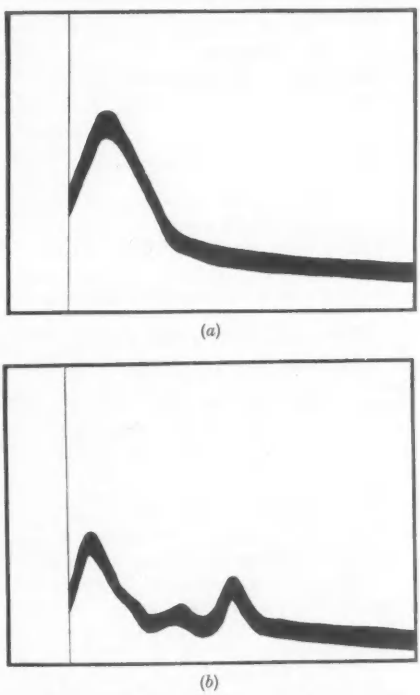

Fig. 2. Sedimentation diagrams of fractions obtained when protoplasts are lysed in 0·3 M-phosphate–salts medium. (*a*) Cytoplasmic fraction. (*b*) Material released from membrane complex when it is washed in 0·01 M-tris + 0·01 M-$MgCl_2$, pH 7·2. Both photographs taken at 16 min. after attainment of full speed. For full experimental details, see text.

Table 1. *Distribution of ribosomal and non-particulate ribonucleic acid in fractions of lysed protoplasts*

The conditions of lysis and methods for chemical analysis were as described in the text.

		Conc. lysis (0·6 M-tris, 0·01 M-$MgCl_2$)		Dil. lysis (0·01 M-tris, 0·01 M-$MgCl_2$)	
		RNA (mg.)	Percentage of total	RNA (mg.)	Percentage of total
Cytoplasmic fraction	RNA in ribosomal form	0·7	1·7	9·4	19·6
	RNA in non-particulate form	19·4	47·2	14·2	29·8
Membrane complex	RNA in ribosomal form	16·8	40·6	18·2	38·1
	RNA in non-particulate form	4·3	10·5	6·1	12·5

Table 2. *Distribution of amino acid-activation enzymes among fractions derived from protoplasts of* Bacillus megaterium

The various fractions were prepared and assayed as described in the Methods section of the paper. The incubation mixtures were buffered with phosphate–salts medium (pH 7·2; final concn. 0·03 M).

Treatment	Fraction	Incorporation of added [^{32}P]pyrophosphate into ATP (%/0·5 mg. of protein)
Protoplasts lysed in 0·3 M-phosphate–salts medium	Cytoplasm	3·1
	5s-fraction of membrane complex	20
	Phospholipoprotein fraction of membrane complex	0·06
Protoplasts lysed in 0·03 M-phosphate–salts medium	Cytoplasm	15
	5s-fraction of membrane complex	29
	Phospholipoprotein fraction of membrane complex	0·04

Table 3. *Effect of ionic strength upon the activity of amino acid-activating enzymes*

The enzyme solution was prepared from a membrane complex after lysis of protoplasts in 0·3 M-phosphate–salts and the activity assayed as described in the Methods section.

	Incorporation of added [^{32}P]pyrophosphate into ATP (%/0·5 mg. of protein)
Enzyme solution made up to volume in 0·01 M-tris	15
Enzyme solution made up to volume in 0·3 M-tris	5
Enzyme solution made up to volume in 0·03 M-phosphate–salts	27
Enzyme solution made up to volume in 0·3 M-phosphate–salts	9

and conc. lysis) can be washed off as ribosomes, 25% as free RNA of low sedimentation constant and approximately 6% is tightly bound to the phospholipoprotein sediment. (Percentage amounts of RNA were calculated from the data presented in Table 1.) The cytoplasmic fraction obtained after dil. lysis contains some ribosomes and amino acid-activating enzymes but is not capable of incorporating [^{14}C]amino acids into protein (Fig. 1; Table 2). No ribosomes and very little [^{32}P]pyrophosphate-exchange activity can be detected in the cytoplasmic fraction obtained by conc. lysis (Fig. 2; Table 2). When, however, a solution of ribosomes suspended in a dilute buffer is dialysed (for 1 hr.) against 0·6 M-tris + 0·01 M-$MgCl_2$ or 0·3 M-phosphate–salts + $MgCl_2$ to saturation, the 30s and 45s peaks disappear and the amount of material absorbing at 260 mμ present in the 4–12s peak increases. It is probable therefore that on conc. lysis the same number of ribosomes are released into the cytoplasmic fraction as on dil. lysis, but the ionic strength is sufficiently high to cause them to break down. The RNA analyses given on Table 1 are consistent with this supposition. The ribosomes that are bound to the membrane complex after conc. lysis must be resistant to the degradative effect of high ionic strength.

The distribution of amino acid-activating enzymes after dil. and conc. lysis is given in Table 2. Table 3 shows that a high concentration of phosphate or tris buffer inhibits [^{32}P]pyrophosphate-exchange activity and the enzyme system is per-

Table 4. *Relative amounts of membrane complex and cytoplasmic fraction obtained after rupturing protoplasts under conditions of conc. lysis and dil. lysis*

The conditions of lysis were as described in the text.

	Dil. lysis (0·03 M-phosphate–salts medium)		Conc. lysis (0·25 M-phosphate–salts medium)	
	% Dry wt. (average of 12 expts.)	Sample figures from 1 expt. (mg.)	% Dry wt. (average of 12 expts.)	Sample figures from 1 expt. (mg.)
Whole protoplasts	100	61·4	100	54·3
Membrane complex	27	15·9	33	18·5
Cytoplasmic fraction	73	45·5	67	35·8

manently damaged as the activity is only partially restored by redilution of the buffer. This explains the low [^{32}P]pyrophosphate-exchange activity of the membrane complex and cytoplasmic fraction after conc. lysis (Table 2).

The proportion of membrane complex and cytoplasmic fraction, expressed as percentage dry weights, when protoplasts are subjected to conc. and dil. lysis are given in Table 4. The small differences are not regarded as significant.

After the ribosomes, free protein and RNA have been washed off a membrane complex, the debris sediments in two clearly defined layers. The bottom layer is heavy, white, opaque and often begins to sediment out on standing. The top layer is brown and gelatinous and requires a fairly high centrifugal force (30 000 rev./min. for 25 min.) to sediment completely, although most of it can be obtained at much lower centrifugal speeds (10 000 rev./min. for 20 min.). The top layer lies loosely on the hard-packed bottom layer and can be clearly separated from it with a spatula. No further amounts of protein, RNA or ribosomes were released when the top and bottom layers were resuspended and thoroughly washed in the presence of ultrasonic vibrations in dilute buffer. Both remained unaltered after repeated resuspension and sedimented with their usual characteristics.

Table 5 shows the proportion of material that is found in the different components of the membrane complex prepared by conc. lysis. The figure given in Table 4 for the ribosomes is somewhat arbitrary, and can be as low as 2 % of the total material of the complex derived from cells in the stationary phase of growth, and as high as 10 % when derived from fresh cells in the logarithmic phase. The proportions of the other components of the complex varies very little from experiment to experiment, being independent of the growth state of the cells and the methods of physical disintegration employed.

Chemical composition of the membrane complex. The analysis of the protein, RNA and lipid present in the various fractions of the membrane complex (prepared by conc. lysis) is given in Table 6, where

Table 5. *Relative proportions by weight of fractions produced by treating the membrane complex (obtained under conditions of conc. lysis) with dilute buffers*

The membrane complex was prepared and fractionated as described in the text.

Fraction	% Dry wt. (average of 15 expts.)	Sample figures from 1 expt. (mg.)
Whole membrane complex	100	38·01
Phospholipoprotein fraction	26	9·85
Mucoprotein fraction	50	19·72
5s-fraction of the complex	16	6·36
Ribosomes	8	3·08

the data were obtained after disrupting the membrane complex by ultrasonic treatment. Closely similar results were obtained in other experiments when the membrane complex was disrupted in simpler ways such as prolonged mechanical shaking in a dilute buffer. The brown gelatinous top layer of the sediment is made up mainly of protein (64 %), lipid (23 %) and RNA (2 %) with a little DNA (1 % by the Ceriotti, 1952, method). More material is insoluble in hot trichloroacetic acid than can be accounted for as protein. Presumably 10–15 % of carbohydrate is also present. The bottom layer contains about 80 % of material insoluble in hot trichloroacetic acid that does not appear to be proteinaceous in nature. It is probable that the bulk of this fraction is made up of carbohydrate, although about 10–20 % of protein is also present. This carbohydrate does not represent cell-wall material, since this is completely digested by the lysozyme treatment (Brookes, Crathorn & Hunter, 1959).

About 70 % of the RNA and 23 % of the protein that can be washed off by dilute buffer from the membrane complex prepared by conc. lysis is accounted for by the ribosomal fraction. The material remaining after separating the ribosomes consists of about 7 % of RNA and 80–85 % of protein, the rest being presumably mainly carbohydrate and inorganic salts. The RNA content of the ribosomes

Table 6. *Chemical composition of components of the membrane complex (prepared by conc. lysis)*

The samples were prepared and analysed as described in the text. The results are expressed as percentages of the total dry weight of each component.

	Phospholipo-protein fraction	Mucoprotein fraction	5s-fraction of the complex	Ribosomes
Material insol. in trichloroacetic acid	74	95	90	40–50
Protein determined by the Lowry method	64	17	87	40–50
Protein determined by the Kjeldahl method	55	9	—	—
RNA by the orcinol method (Ceriotti, 1952)	1–2·5	0·5–1	—	50–65
RNA from the u.v. absorption at 260 mμ	2	1	7	50–60
Lipid from the wt. extracted by chloroform–ethanol	23–25	0–3	—	—
Phospholipid from the phosphorus content of the same extract	19	0·5–1	—	—

varies between 50 and 70 %, but most preparations contain 55–60 % of RNA and 40–45 % of protein.

For the remainder of the paper, the top layer of the sediment will be termed the 'phospholipoprotein fraction' and the bottom layer the 'mucoprotein fraction'. The free protein and RNA left after the ribosomes have been removed from the supernatant fraction will be termed the '5s-fraction of the complex'.

Electron microscopy of the fractions of the membrane complex. Under the electron microscope, the cytoplasm of sections of whole cells of *B. megaterium* is seen to contain abundant 150 Å particles, and to be surrounded by a double membrane underlying the cell wall (Plate 1*a*). Protoplasts present a similar picture, except for the absence of the cell wall.

The unwashed membrane complex obtained by conc. lysis of protoplasts (Plate 1*b*) is made up of a mixture of large, fairly empty vesicles bounded by a double membrane, smaller dense vesicles (in some sections, the preparation consists entirely of these smaller vesicles) and some strands that may represent DNA; 150 Å particles can again be seen, and are almost exclusively attached to the small dense vesicles. The phospholipoprotein fraction (Plate 1*c*) obtained by washing in dilute buffer the membrane complex prepared by conc. lysis consists entirely of very small membranous vesicles. These vesicles are smaller than any that are seen in the unwashed membrane complex and are often only 300–400 Å in diameter. No 150 Å particles are visible in this fraction. The well washed mucoprotein fraction (Plate 1*d*) is seen to consist of twisted irregular filaments, together with some fluffy diffuse material, and again no 150 Å particles are visible. Electron micrographs of isolated ribosomes show normal ribonucleoprotein particles.

Incorporation of [^{14}C]amino acids into components of the membrane complex

The membrane complex produced by conc. lysis of protoplasts can be actively incorporate [^{14}C]-amino acids into proteinaceous material when suspended in 0·5 M-phosphate–salts medium under conditions described by Hunter *et al.* (1959). As mentioned above, the membrane complex obtained by dil. lysis is inactive in this respect. In some experiments, the isolated membrane complex was used for incorporation studies, but in most of the more recent work protoplasts were preferred as less manipulation was required to obtain the fractions.

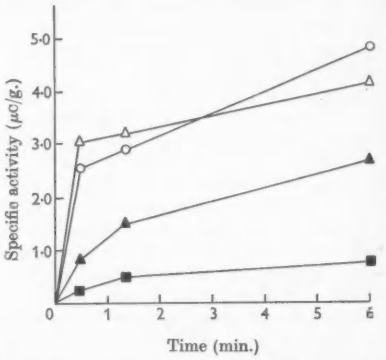

Fig. 3. Incorporation of L-[^{14}C]phenylalanine into the proteins of the components of the membrane complex after incubation of the isolated complex in 0·5 M-phosphate–salts medium. Labelled amino acid (10 μc) was added to membrane complex derived from 1·5 g. dry wt. of whole cells which were suspended at a final density of 2 mg./ml. Samples were taken at various times and fractionated and analysed as described in the Methods section. △, Phospholipoprotein; ▲, ribosomes; ○, 5s protein; ■, mucoprotein.

The pattern of labelling of the isolated membrane complex during short exposures to [^{14}C]amino acids is illustrated in Fig. 3. In this experiment, the membrane complex was labelled with L-[^{14}C]-phenylalanine and samples were taken at 25, 80 and 360 sec. It can be seen that, at early times, the specific radioactivity of the protein of the phospholipoprotein fraction exceeds that found in the protein of the 5s-fraction of the complex, but, at later times, these specific activities are reversed. In

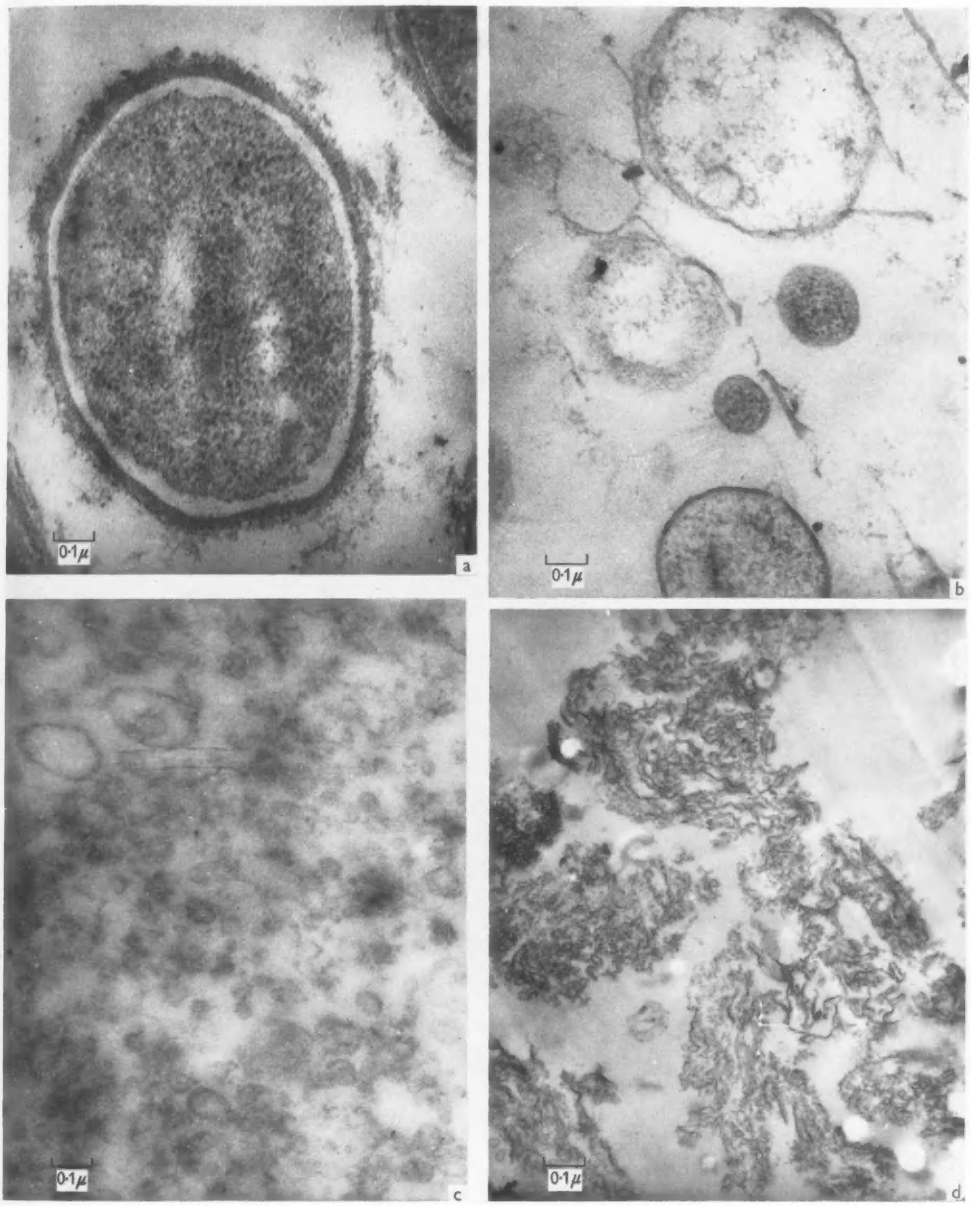

Plate 1. Electron micrographs of sections prepared as described by Mercer (1959) from: (*a*) whole cell of *Bacillus megaterium*; (*b*) a pellet of the membrane complex, prepared by conc. lysis of protoplasts, as described in the text; (*c*) the phospholipoprotein fraction; (*d*) the mucoprotein fraction; (*c*) and (*d*) were prepared by fractionating the membrane complex as described in the text.

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longer incubations (up to 2 hr.), the specific radioactivity of the protein in the phospholipoprotein fraction tends to remain the same whereas that of the 5s-fraction continues to rise. At very early times (5–30 sec.) the specific radioactivity of the ribosomal protein (Figs. 3 and 4; Table 7) is either lower or about the same as that of the phospholipoprotein, a fact that has been confirmed in numerous experiments. The purity of the ribosomal fraction was checked by rerunning 30s and 45s particles from the density-gradient separation in the ultracentrifuge and also by determinations of the RNA:protein ratio. It is certain that the ribosome fraction obtained in these experiments is uncontaminated and made up only of 30s and 45s particles.

In the experiment illustrated in Figs. 4 and 5, protoplasts were labelled with L-[^{14}C]threonine for 5 sec., 15 sec. and 2 min. The plot of the specific activities of the protein fractions (Fig. 4) shows that the course of labelling is similar to that observed in the isolated membrane complex, except that the highest specific activities eventually attained are now found in the cytoplasmic fraction. The plot of the same results in terms of total radioactivities (Fig. 5) illustrates well the fact that at early times most of the label accumulates in the phospholipoprotein fraction and then seems to be passed on to the cytoplasmic fraction, possibly through the protein of the 5s-fraction of the complex. The total activity accumulating in the 5s-fraction of the complex is lower in protoplast experiments than in experiments with the isolated membrane complex, and some of the 5s-fraction probably consists of cytoplasmic precursors. There is less protein in the ribosomal than in the phospholipoprotein fraction and consequently the total radioactivity of the ribosomal protein is much lower than that of the phospholipoprotein fraction in a lot of this sort (Fig. 5). At all times, the labelling of the mucoprotein fraction is very low, even though it must be contaminated to a small extent by the phospholipoprotein fraction.

In summary, these experiments, which have been repeated many times with similar results, gave an indication that labelled protein, appearing first in the lipoprotein and ribosomal fractions, was passed on in sequence from the lipoprotein to the 5s-fraction of the complex and then to the cytoplasmic protein. The mucoprotein fraction, however, appeared to be inert. Further evidence in favour of this postulated sequence of events was provided by two types of 'chaser' experiments, in which the addition of the labelled amino acid to protoplasts was followed after a short time by a 'chase' of a large amount of the same unlabelled amino acid. Table 7 illustrates the changes in specific radioactivities of the various protein fractions when protoplasts were labelled for a very short time (10 sec.) and then 'chased' for 2 min. with a large amount of unlabelled amino acid. The specific radioactivity of the lipoprotein, initially relatively

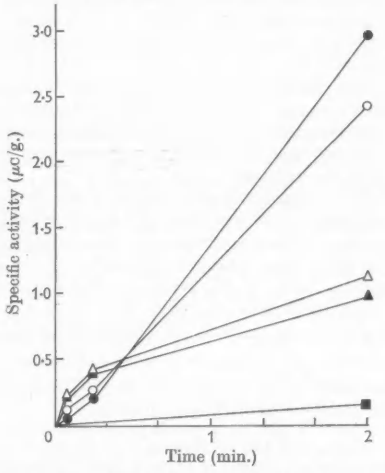

Fig. 4. Incorporation of L-[^{14}C]threonine into the protein of the cytoplasmic fraction and components of the membrane complex of protoplasts of *Bacillus megaterium*, suspended in 0·5M-phosphate–salts medium at a density of 50 mg./ml. Labelled amino acid (12 μc) was added to protoplasts derived from 1·5 g. dry wt. of whole cells. The samples were taken at various times and the fractions prepared and analysed as described in the Methods section. △, Phospholipoprotein; ▲, ribosomes; ○, 5s protein; ■, mucoprotein; ●, cytoplasmic fraction.

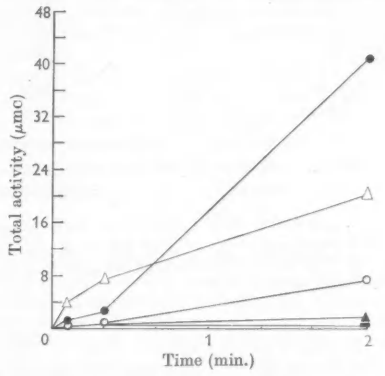

Fig. 5. Results of the experiment described in Fig. 4 plotted in terms of the total radioactivity present in each fraction. △, Phospholipoprotein; ▲, ribosomes; ○, 5s protein; ■, mucoprotein; ●, cytoplasmic fraction.

high, falls during the course of the 'chase', whereas the protein of the 5s-fraction and cytoplasm rises in activity. In this experiment, the specific radioactivities of both the 30s and 45s ribosomes was low. Table 8 illustrates an experiment where the 'chaser' dose was added after the incubation with the [^{14}C]amino acid had proceeded for 2 min., and the specific activities of the protein fractions were measured at intervals afterwards. Although the radioactivity in the lipoprotein fraction did not by any means drop to zero, it decreased progressively whereas the protein of the 5s-fraction of the complex and the cytoplasm gained a similar amount of label. In experiments of this type with rather longer times, the specific and total radioactivities found in the ribosomal protein are low.

Table 7. *Transfer of* L-[^{14}C]*phenylalanine incorporated into the protein of the components of the membrane complex and the cytoplasmic fraction of protoplasts of* Bacillus megaterium

B. megaterium were suspended in 0·5M-phosphate–salts medium after the addition of unlabelled L-[^{12}C]phenylalanine. Protoplasts, derived from 0·7 g. dry wt. of whole cells and suspended at a density of 40 mg./ml., were incubated with 0·15 μc of L-[^{14}C]amino acid for 10 sec. and then 16 mg. of 'chaser' L-[^{12}C]amino acid was added. For other details see Methods section.

Fraction	Specific activity after labelling for 10 sec. with [^{14}C]amino acid (μmc/g.)	Specific activity after labelling for 10 sec. with [^{14}C]amino acid followed by 'chasing' for 2 min. with [^{12}C]amino acid (μmc/g.)
Phospholipoprotein	0·194	0·11
5s-fraction	0·12	0·17
Cytoplasmic fraction	0·034	0·10
30s ribosomes	0·013	0·013
45s ribosomes	0·056	0·050

DISCUSSION

The fractionation of the membrane complex described above enables its main constituents to be identified and their functions examined. Comparisons can also be made with observations on other cell types. It is now established that the membrane complex prepared by the lysis of protoplasts with comparatively concentrated salt solutions contains ribosomes, which can be detached by washing with buffers of low ionic strength. There is no conflict here with the findings of other workers (e.g. Tissières & Watson, 1958; McQuillen, Roberts & Britten, 1959), who have obtained ribosomes in cytoplasm. Their methods have all involved the use of media of low ionic strength, and in our experiments, also, these conditions lead to the appearance of ribosomal particles in the cytoplasmic fraction. In media of high ionic strength, ribosomes detached from the membrane complex are broken down, possibly because of the removal of magnesium ions. The ribosomes that remain attached to the membrane complex under these conditions are not degraded, perhaps because they are present in the more diffuse state suggested by Hanzon, Hermodsson & Toschi (1959) to be the true physiological form of the ribosome. However, the electron micrographs of sections of both the whole cell and membrane complex show large numbers of particles 100–150 Å in diameter, and they can be obtained in the usual dense form after lysis. It is also remarkable that the activity of the membrane complex is maximal in a medium that would break down free ribosomes, presumably a further indication that the active ribosomes are bound in a special way to the membrane complex.

The solubilized extract containing the ribosomes also contains much material of 4–6s. This includes the amino acid-activation enzymes and also a quantity of RNA that is capable of binding amino acids in a form labile to the action of alkali and ribonuclease (Hunter *et al.* 1959). This RNA fraction probably corresponds to the soluble RNA fraction

Table 8. *Transfer of* L-[^{14}C]*leucine incorporated into the protein of the components of the membrane complex and the cytoplasmic fraction of protoplasts of* Bacillus megaterium

B. megaterium were suspended in 0·5M-phosphate–salts medium, after the addition of L-[^{12}C]amino acid. Protoplasts, derived from 1·5 g. dry wt. of whole cells, were suspended at a density of 50 mg./ml. and incubated with 1·5 μc of L-[^{14}C]leucine for 2 min. and then 40 mg. of 'chaser' L-[^{12}C]leucine was added. For other details see Methods section.

Fraction	Total radioactivity (μmc) 2 min. after adding [^{12}C]leucine	4 min. after adding [^{12}C]leucine	12 min. after adding [^{12}C]leucine
Phospholipoprotein	15·0	12·3	9·5
5s-fraction	7·4	9·3	10·8
Cytoplasm	47	52	54
Ribosomes	0·53	0·53	0·53

of rat liver, but it is not clear at present whether it differs essentially from the cytoplasmic RNA removed previously.

The material that remains readily sedimentable after treating the membrane complex with dilute buffers is obtained in two layers which can easily be separated. The lower heavy layer or mucoprotein fraction contains little or no lipid or RNA and consists largely of carbohydrate with some protein. It appears mainly as large irregular fragments in electron micrographs. The phospholipoprotein fraction that lies above it also seems to be free from ribosomes, and indeed the low content of RNA, apart from the evidence derived from electron microscopy, would make it unlikely that an appreciable number could be present. It is not clear whether the phospholipoprotein fraction is derived entirely from the cytoplasmic membrane of the cell, or whether some of it originates in cytoplasmic structures associated with ribonucleoprotein. It is seen in the electron microscope as very small spherical vesicles often only 300–400 Å in diameter. The mucoprotein and phospholipoprotein fractions obtained by us are probably related to the fractions described by Weibull & Bergström (1958), although the experimental procedures outlined here enable the two fractions to be differentiated more clearly.

The data obtained when the membrane complex is fractionated after a preliminary incubation of the complex or protoplasts with labelled amino acids are rather complex. One fact stands out very clearly: the mucoprotein fraction is quite inert in the labelling process, confirming that our preparations were almost completely free from whole cells, since any whole cells present would have been obtained in this fraction. Labelled protein very soon appears in the 5s-fraction of the complex, but the results that have been obtained, and, in particular, the evidence of transfer of ^{14}C-labelled protein to this fraction obtained in the 'chaser' experiments, make it unlikely that any labelled protein is independently formed here. The primary site of formation of labelled protein thus seems to be in the phospholipoprotein fraction or in the ribosomes. Where the exposure to labelled amino acids has been carried out for short times only, the phospholipoprotein fraction always contains protein labelled at comparable or higher specific activity than that observed in the ribosomes. On the other hand, the total radioactivity in the protein of the phospholipoprotein fraction is considerably higher than that found in the ribosomes. If these ribonucleoprotein particles were the primary source of labelled protein and passed their nascent protein very rapidly on to the phospholipoprotein, then it would be expected that the 'chaser' experiments would show a very marked fall in the specific radioactivity of the ribosomes as their labelled nascent protein was replaced by practically non-radioactive material. However, the ribosomal protein was not observed to fall much in specific radioactivity during these experiments, and it is difficult to avoid the conclusion that the phospholipoprotein is labelled independently or concurrently.

Experiments carried out *in vivo* (e.g. McQuillen *et al.* 1959; Siekevitz & Palade, 1960) have been interpreted as indicating that the primary source of labelled protein is the ribosomal particle. However, the methods used by the latter workers for the preparation of the ribosomal particles were such that delicate phospholipoprotein structures would have been disrupted, and any freshly formed protein bound to them might have been released and attached to the ribosomes. The same criticism might apply in part also to the former group of workers (McQuillen *et al.* 1959), but in any case their results clearly show (especially their Fig. 6) that some material containing protein of high specific radioactivity sediments more rapidly than the ribosomes. This material may be the equivalent of our phospholipoprotein fraction, although most of the more readily sedimentable material was removed in their experiments and not examined at all. Where ribosomes have been obtained under conditions such that any associated phospholipoprotein would remain intact, they are often capable of very active protein biosynthesis (e.g. Webster, 1959). However, in the pea-seedling ribosome (Ts'O & Sato, 1958) the most highly labelled fraction again appears to be a phospholipoprotein. A ribosomal-free debris fraction has been obtained from *Escherichia coli* (Schachtschabel & Zillig, 1959; McCorquodale & Zillig, 1959) that is very active in incorporating amino acids into protein, and Dr P. N. Campbell (personal communication) has obtained evidence that serum albumin synthesis in rat-liver microsomes is located solely on the membrane part of the microsome.

There is thus considerable evidence for the implication of phospholipoprotein as well as the ribosomes in at least the final stages of protein biosynthesis. A possible mechanism to account for this has been suggested elsewhere (Hunter & Godson, 1961).

SUMMARY

1. The membrane complex obtained by lysing protoplasts of *Bacillus megaterium* in buffers of physiological strength is disrupted by exposure to buffers of low ionic strength.

2. Under these conditions, the following fractions are obtained and separated cleanly from each other: (1) a mucoprotein fraction; (2) a phospholipoprotein fraction, seen in the electron microscope as very small spherical vesicles free from ribosomes; (3) ribonucleoprotein particles (ribo-

somes of 30s and 45s); (4) ribonucleic acid of low molecular weight, together with solubilized protein that includes all the amino acid-activation enzymes of the complex.

3. When the whole membrane complex or protoplasts are incubated with labelled amino acids, labelled protein appears first in the ribosomal and phospholipoprotein fractions. The greater part of this labelled protein is found in the phospholipoprotein fraction, and there is no evidence that this is derived from the ribosomes.

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Enzymic Desulphation of L-Serine *O*[^{35}S]-sulphate: the Intracellular Localization of the Enzyme

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Dodgson, Lloyd & Tudball (1961*a*, *b*) reported that an enzyme system, capable of liberating inorganic [^{35}S]sulphate from L-serine *O*[^{35}S]-sulphate, is present in rat liver. The enzyme system could be extracted from fresh rat-liver suspensions with water. Studies on the localization of the enzyme system in the rat-liver cell and its distribution in other rat tissues and in other species have now been made.

MATERIALS AND METHODS

L-Serine O[^{35}S]*-sulphate.* This was prepared and diluted with carrier L-serine *O*-sulphate as described by Dodgson *et al.* (1961*b*).

Enzyme sources. Pig, sheep, chicken and ox livers and ox pancreas were slaughterhouse specimens. Human liver was a post-mortem sample provided by the Department of Pathology. Periwinkles (*Littorina littorea*) and limpets (*Patella vulgata*) were collected at low tide from the beach at Sully, Glamorgan. Snails (*Helix pomatia*) were purchased from L. Haig, Beambrook, Newdigate, Surrey. Brewer's yeast was kindly provided by Brain's Brewery, Cardiff. Salmon (*Salmo salar*) liver was obtained from the fish-market. Frogs (*Rana temporaria*) were kindly supplied by the Department of Zoology. Taka-diastase (lactose-free) was a gift from Parke, Davis and Co. Ltd., and acetone-dried cells of *Alcaligenes metalcaligenes* (see Dodgson, Melville, Spencer & Williams, 1954) were prepared at the M.R.C. Antibiotic Research Station, Clevedon.

Fractionation of cellular components of rat liver. M.R.C. hooded rats were used throughout. Fractionations of cellular components in sucrose media were carried out according to the procedures of Hogeboom, Schneider & Pallade (1948) (hyperosmotic sucrose, 0·88M) and Schneider (1948) (isosmotic sucrose, 0·25M). The separated fractions were resuspended in the appropriate sucrose medium for enzyme assay. Preliminary experiments showed that sucrose did not interfere with the assay method.

Uncontaminated cell nuclei. These were obtained by method 1 of Roodyn (1956). For enzyme assay the nuclei were resuspended in the fractionation medium (0·25M-sucrose containing 1·8mM-$CaCl_2$), which did not interfere with the assay method.

Determination of enzyme activity

A procedure based on that described by Dodgson *et al.* (1961*b*) was used. For the assay of whole tissue suspensions 50μl. of 0·02M-L-serine *O*[^{35}S]-sulphate in 0·5M-tris–acetic acid buffer, pH 7·0, was incubated with an equal volume of the enzyme preparation. These volumes were doubled for the assay of the crude cell-nuclei fractions and halved for all other enzyme assays. Except where otherwise stated, incubation was for 3 hr. at 38°. Enzyme action was stopped by placing the reaction tubes in boiling water for 30 sec. Control determinations consisted of substrate and enzyme incubated separately and mixed immediately before the boiling-water treatment. Precipitated protein was removed by centrifuging and 20μl. of the clear supernatant was applied as a spot (7 mm. diameter, prepared by alternate spotting and drying) to Whatman no. 1 paper and subjected to electrophoresis for 2 hr. at a potential gradient of 12v/cm. in the presence of 0·1M-ammonium acetate. Tests, controls and markers of L-serine *O*[^{35}S]-sulphate and inorganic $^{35}SO_4^{2-}$ ions ($Na_2{}^{35}SO_4$) were run on the same paper. After drying and cutting into suitable strips, radioactive zones were detected with the C. 100 Actigraph automatic strip-scanner (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) as described by Dodgson *et al.* (1961*b*). The areas of the peaks on the recording chart (corresponding to enzymically liberated inorganic $^{35}SO_4^{2-}$ ions and residual L-serine *O*[^{35}S]-sulphate) were cut out and weighed on a microbalance, and the weights used to calculate enzyme activity.

The following checks on the validity and limitations of the method were made.

Variation in weights of standard areas of recording chart paper. Standard areas (varying between 4 and 17·5 cm.^{2}) were cut from different regions of the recording chart paper and weighed. The variation in the weights of identical areas was less than $\pm$2·5%.

Reproducibility of the electrophoresis procedure. Variations in the count rate (and hence of areas of peaks on the recording chart) of identical amounts of radioactively labelled material could arise as a result of the electrophoresis procedure (e.g. from uneven spreading). This possibility was checked by subjecting several 20μl. samples of the substrate solution to paper electrophoresis. Within the limits imposed by variations in the density of the chart paper, the weights of the areas of the peaks of radioactivity obtained were reproducible.

In assaying the activity of different enzyme preparations, variations in the amounts of product liberated and of substrate remaining are to be expected. It was therefore necessary to show that the amounts of product (or substrate remaining) and the areas of the peaks on the recording chart were linearly related. Inorganic $^{35}SO_4^{2-}$ ions or L-serine *O*[^{35}S]-sulphate, in various concentrations and representing 0–100% desulphation of substrate, were subjected to electrophoresis and the strips analysed. In each case a linear relationship between concentration and peak area was obtained.

As a final check on the electrophoresis procedure, standard amounts of inorganic $^{35}SO_4^{2-}$ ions and L-serine *O*[^{35}S]-sulphate were subjected to electrophoresis, separately and as mixtures. The areas of the peaks obtained with the mixtures were identical with those obtained when the components were run separately.

Recovery of radioactivity from enzyme preparations. The recovery of radioactivity from incubation mixtures of enzyme and substrate was always checked by means of an additional control experiment in which water was substituted for the enzyme preparation. The total radioactivity recovered from the test and normal control determinations could then be compared with that recovered in the absence of enzyme preparation. Table 2 shows that recoveries from enzyme preparations were somewhat variable but were sufficiently good for the purpose of the present study.

EXPERIMENTAL AND RESULTS

Fractionation of intracellular components of rat liver

Table 1 shows the activities of the various cellular fractions obtained by simple differential centrifuging of rat-liver suspensions in hyper- and iso-osmotic sucrose solutions. Enzyme activity was concentrated mainly in the high-speed supernatant fraction although appreciable activity was also present in the so-called 'nuclear' fraction. However, microscopic examination of this fraction showed the presence of unruptured cells and relatively little enzyme activity could be detected in uncontaminated nuclei isolated by the procedure of Roodyn (1956). The low enzyme activity associated with the 'mitochondrial' and 'microsomal' fractions is probably not significant and no attempt was made to resolve these fractions further by density gradient centrifuging procedures.

Comparative activities of male and female rat livers

During studies on the microsomal arylsulphatase of rat liver, Dodgson, Spencer & Thomas (1953) noted that the livers of male rats were almost twice as active as those of females. The relative activities of male and female rat livers towards L-serine *O*[^{35}S]-sulphate were checked as follows. The livers of five male and five female rats were weighed and squeezed through fine (1 mm.^{2}) brass-wire meshes in order to remove connective tissue. The pulps were suspended with a glass homogenizer in sufficient ice-cold water to give a final concentration of 10% (w/v), and the activity of each suspension was

determined as described earlier. The liberation of inorganic $^{35}SO_4^{2-}$ ions, expressed as a percentage of the available ^{35}S, averaged 33·3 % (range 32·1–35·9 %) for male rats, whereas the corresponding figures for females was 26·8 % (range 22·5–30·6 %). It is doubtful whether these differences are significant in view of the relatively small numbers of animals tested.

Distribution of enzyme activity in other rat tissues and in other species

Except where otherwise stated, a 20 % (w/v) suspension of the tissue in ice-cold water was frozen and thawed, resuspended, and centrifuged for 20 min. at 0° and 2000*g*. The activity of the supernatant was then determined as described pre-

Table 1. *Enzymic liberation of inorganic $^{35}SO_4^{2-}$ ions from* L-*serine* O[^{35}S]-*sulphate by fractions obtained by the differential centrifuging of rat-liver suspensions in sucrose solutions*

See text for experimental details. The enzyme activities of the fractions are expressed as percentages of that obtained with the whole-liver suspension. Ranges are given in parentheses.

Fractionation medium	No. of experiments	Fraction	Activity (%)
Hyperosmotic sucrose	7	Nuclei	21·8 (14·5–32·0)
		Mitochondria	5·1 (0–13·0)
		Microsomes	8·3 (0–15·9)
		Final supernatant	58·0 (43·6–71·6)
Iso-osmotic sucrose	1	Nuclei	37·0
		Mitochondria	5·8
		Microsomes	4·5
		Final supernatant	68·0
Iso-osmotic sucrose –$CaCl_2$	3	Uncontaminated nuclei*	2·5 (0–7·6)

* Prepared by the method of Roodyn (1956).

Table 2. *Liberation of inorganic $^{35}SO_4^{2-}$ ions from* L-*serine* O[^{35}S]-*sulphate by aqueous extracts of rat tissues and of other organisms*

See text for experimental details.

Species	Tissue	Initial concn. of tissue suspension (%, w/v)	Incubation period (hr.)	Percentage of available ^{35}S liberated as inorganic sulphate	Recovery of ^{35}S from incubation mixture (%)
Rat	Kidney	20	22	31·3	93·7
	Heart	20	22	0	96·2
	Spleen	20	22	< 5·0	96·5
	Lung	20	18	< 5·0	96·0
	Pancreas	20*	18·5	25·3	85·5
	Small intestine	20	3·5	0	90·3
Ox	Liver	20	3	63·1	—
	Pancreas	20	3	31·0	106·2
Human	Liver	20	4	22·0	86·7
Mouse	Liver	10	3	15·0	105·0
Pig	Liver	15	3	46·6	103·4
Sheep	Liver	20	3	54·1	—
Chicken	Liver	10	4	39·9	—
Frog (*Rana temporaria*)	Liver	20*	3	6·5	94·7
Salmon (*Salmo salar*)	Liver	20*	3	40·3	93·8
Snail (*Helix pomatia*)	Digestive juice	†	20·5	0	101·1
Limpet (*Patella vulgata*)	Digestive gland	20	2·5	0	88·9
Periwinkle (*Littorina littorea*)	Digestive gland	20	2·5	0	96·9
Taka-diastase (*Aspergillus oryzae*)	—	5*	4	0	95·0
Alcaligenes metalcaligenes	—	5*	4	0	88·5
Baker's yeast	—	20	3	0	105·0
Brewer's yeast	—	20*	18	< 5·0	97·3

* Whole tissue suspensions used. † Undiluted juice dialysed for 16 hr.

viously, although in some cases a long incubation period was used (see Table 2).

The enzyme was present in the livers of all the mammalian species examined and was also found in rat kidney and pancreas and in ox pancreas. Appreciable activity was also obtained with chicken and fish (salmon) livers but not with frog liver or with any of the lower organisms that were examined.

DISCUSSION

Desulphation of L-serine *O*[^{35}S]-sulphate can be achieved by mammalian, avian and fish liver extracts, and, although the responsible enzyme system does not appear to be particularly active, this view must be accepted with reserve until the optimum conditions for activity have been completely defined. Dodgson *et al.* (1961*b*) have pointed out that the desulphation may not necessarily be due to a simple hydrolase (a sulphatase), but preliminary efforts to establish the exact mechanism of the process have been hindered by the crude nature of the rat-liver preparations. Certainly no known sulphatase can be responsible for the activity. Chondro-, glyco- and myro-sulphatase do not occur in rat livers (Dohlman, 1956; Dodgson & Lloyd, 1961; Baum & Dodgson, 1957) and cellulose polysulphatase (found in at least one mollusc) is apparently specific for β-linked polyglucans containing sulphate in the 2- or 3-position of the glucose residues (Takahashi & Egami, 1961). Cortisone 21-sulphatase occurs in the digestive juice of the snail (*Helix pomatia*) but L-serine *O*-sulphate is not a substrate for the enzyme (Dodgson, 1961). 3β-Steroid sulphatase and arylsulphatases A, B and C do occur in mammalian livers but are localized in the particulate material of the liver cell (Roy, 1957, 1960; Dodgson, Spencer & Thomas, 1953, 1955).

Neuhaus & Byrne (1959) and Borkenhagen & Kennedy (1959) have shown that rat and chicken livers possess a phosphatase that is active towards L-serine *O*-phosphate (*O*-phosphoserine). A similar enzyme is also present in baker's yeast (Schramm, 1958). No enzyme activity towards L-serine *O*[^{35}S]-sulphate could be detected in baker's yeast in the present study, nevertheless the possibility that the same enzyme is attacking both substrates cannot yet be excluded.

The occurrence, in proteins, of L-serine *O*-phosphate is well known but the presence of the corresponding sulphate ester has never been demonstrated. Free L-serine cannot be sulphated enzymically by particle-free rat-liver preparations which are able to sulphate steroids, phenols, amines and simple alcohols (Spencer, 1960; Dodgson *et al.* 1961*b*), but this does not exclude the possibility that protein-bound serine residues can be sulphated. Free L-tyrosine cannot be sulphated by rat-liver preparations (see, for example, Grimes, 1959) but occurs in fibrinogen as the corresponding *O*-sulphate ester (Bettelheim, 1954). It may be of significance that the enzyme systems responsible for the desulphation of L-serine *O*[^{35}S]-sulphate and for the sulphation of hydroxylated compounds co-exist in the cell sap. Attempts to achieve the enzymic sulphation of free L-serine have therefore always been made with enzyme preparations that contain the desulphating system.

SUMMARY

1. Further observations have been made on a method of determining the activity of the rat-liver enzyme system which is able to desulphate L-serine *O*[^{35}S]-sulphate.

2. The desulphating enzyme is mainly localized in the cell sap of the liver cell. Its distribution in the cell differs from that of any known mammalian sulphatase enzyme.

3. The enzyme is present in human, ox, sheep, pig, mouse, chicken and salmon livers, but significant amounts could not be detected in a number of lower organisms that were examined.

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Gangliosides and Related Substances of Isolated Cerebral Tissues Examined in Relation to Tissue Excitability

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When cerebral tissues are kept in cold media they lose their normal metabolic response to applied electrical pulses (Marks & McIlwain, 1959). Response can be restored by incubating with tissue extracts and blood-plasma fractions, and the activity of such fractions has been correlated with their content of neuraminic acid derivatives (McIlwain, 1960*a*, 1961*a*). The most potent of these derivatives examined were the cerebral gangliosides, and it has therefore been considered that more might be learned about cerebral excitability by measuring the quantity of gangliosides and of related substances in the brain and in isolated cerebral tissues prepared and maintained in different fashions, including especially those kept in cold media and incubated with various additional substances.

The gangliosides (for nomenclature see McIlwain, 1961*c*) contain fatty acids, sphingosine, *N*-acetylgalactosamine and glucose as well as the *N*-acetylneuraminic acid by which they are most frequently determined (Svennerholm, 1957; Klenk & Gielen, 1960; Rosenberg & Chargaff, 1960; van Heyningen & Miller, 1961); they comprise more than one molecular species (Meltzer, 1959; Folch & Lees, 1959; van Heyningen & Miller, 1961). Cerebral tissues also contain derivatives of *N*-acylneuraminic acids (sialic acids) which either are not gangliosides or are further derivatives or complexes of gangliosides (Svennerholm, 1956; Rosenberg & Chargaff, 1958; and see below), and the particular distribution of gangliosides between aqueous salt solutions and non-aqueous solvents, investigated by Folch, Lees & Sloane-Stanley (1957), gives valuable means of obtaining additional specificity in methods for their determination. Long & Staples (1959) applied such distribution in a method for separating and determining the gangliosides, which forms the basis of that studied below. We have, however, given further attention to two aspects of the determination: (1) the completeness of extraction of the lipid-soluble neuraminic acid derivatives; (2) the use of an alternative spectrophotometric method for the measurement, namely the thiobarbiturate method of Warren (1959), as well as the ferric chloride–orcinol reaction as used by Long & Staples (1959).

EXPERIMENTAL

Tissues and metabolic experiments

Cerebral tissues. (*a*) For tissue rapidly fixed *in situ*, young rats were projected head first into approx. 400 ml. of liquid N_2 in a vacuum jar. After about 5 min., when vigorous bubbling had ceased, the animal was removed with cold tongs, wrapped in a cloth with its head projecting, fur was scraped from the head with a scalpel and the skull removed with a chisel also cooled in the liquid N_2. The brain was chiselled out from the frozen animal on to a pad of clean paper and quickly transferred to a cold glass-stoppered tube.

(*b*) Blocks of tissue removed at room temperature were obtained by stunning guinea pigs or rats by a blow on the back of the neck, exsanguinating, opening the skull, removing the brain and taking a sample with a scalpel within 3 min. of stunning the animal.

(*c*) Slices for incubation were cut from the brain removed as in (*b*), with a strip of razor blading and a recessed guide as described by McIlwain (1961*b*). The incubating medium (below) was used to lubricate the blade in cutting and slices (0·35 mm. in thickness) were obtained, floated free in a dish of medium, picked out with a wire rider, drained by repeated contact with a glass surface and weighed.

Media and incubation. All media contained (mM): NaCl, 134; KCl, 5·4; $CaCl_2$, 2·6; $MgSO_4$, 1·3; KH_2PO_4, 1·2; glucose, 10. In addition, the phosphate medium contained 10·4 mM-Na_2HPO_4 brought to pH 7·4 by HCl; the tris medium contained 25 mM-2-amino-2-hydroxymethylpropane-1:3-diol (brought to pH 7·4 by HCl) and the glycylglycine medium contained 30 mM-glycylglycine (brought to pH 7·4 by NaOH). Media were prepared from stock solutions immediately before use, oxygenated, distributed among conical manometric vessels of 15–20 ml. and any further additions made as indicated in individual experiments mentioned below. The tissues (described above) were added immediately after weighing; the vessels were equilibrated with O_2 and shaken at 37·5°.

In Expts. 6–8 of Table 5, electrical pulses were applied to slices incubated in silver-grid electrodes *H* in vessels *A* (Ayres & McIlwain, 1953; McIlwain, 1960*b*). Alternating pulses of exponential time–voltage relationship, time-constant 0·4 msec. and 10 v peak potential were applied at 100/sec.

Determination of ganglioside-like extractives

Extraction. Procedure *A*. Weighed specimens (80–100 mg.) were placed at 0° in homogenizer tubes, 150 mm. × 12 mm., provided with pestles ground smoothly to fit. From a $CHCl_3$–methanol mixture (2:1, v/v; also at 0°)

volumes (ml.) 19 times the weight of the tissue (g.) were pipetted into the tubes; the tissue was ground in the solvent and the mixture left for 3 min.

Extraction in the presence of added substances. In the experiments of Table 2, the additional substances were dissolved or suspended in 10 times their weight of water and added to the homogenizer tubes of procedure *A*. The weighed slices were then added and ground at 0° with the fluid for 3 min. or at intervals for 30 min. according to the experiment. The $CHCl_3$–methanol mixture, of volume 19 times the weight of tissue plus added water, was then pipetted into the tube and its contents were ground with the pestle. When the additional substance was suramin, used at 5 mg./100 mg. of tissue, the extraction is termed procedure *B*.

Extraction of fluids. From vessels after incubation, portions (2 ml.) of fluids were pipetted into 10 ml. beakers and evaporated to dryness over $CaCl_2$ *in vacuo*. The residue was rubbed up in 0·25 ml. of water or of a suramin solution, and ground with 19 vol. of $CHCl_3$–methanol (2:1, v/v).

Solvent partition. This procedure is based on those of Folch *et al.* (1957) and Long & Staples (1959). The $CHCl_3$–methanol tissue suspensions or fluid extracts were filtered by gravity through fluted circles of Whatman paper no. 43 (7 cm. diam.) into graduated 125 mm. × 16 ml. glass-stoppered tubes. The volumes of the filtrates were noted, 0·2 vol. of 0·1 M-KCl was added to each and the tubes were shaken thoroughly so that an emulsion was maintained for 10–15 min. The tubes were then centrifuged at 1200*g* for 3 min. and the upper layers (*a*) collected by Pasteur pipette. To the lower layers were added 0·4 vol. of $CHCl_3$–methanol–0·1 M-KCl (3:48:47, by vol.). The tubes were shaken and centrifuged as before, and their upper layers combined with layers (*a*). (A further extraction contributed less than 3% of additional chromogen, and was not normally performed. Filtration under the conditions specified removes non-ganglioside neuraminic acid-containing substances by absorption, a process to be reported in detail subsequently.)

Acylneuraminic acid determinations. (i) Bial's $FeCl_3$–orcinol reaction. This was close to the method of Böhm, Dauber & Baumeister (1954) as applied to cerebral tissues by Long & Staples (1959); Bial's orcinol reagent was made by dissolving 200 mg. of orcinol in 4 N-HCl, adding 1 ml. of 2% (w/v) $FeCl_3$ and making to 100 ml. with 4 N-HCl.

The upper layers after partition were evaporated to dryness in 125 mm. × 16 mm. tubes, in a stream of N_2 in a water bath at 80°. To the residues was added 2 ml. of 4 N-HCl, the tubes were shaken and 1 ml. was removed from each to a second set of similar tubes. One set of tubes was heated at 100° for 30 min. These constituted the control tubes; *N*-acetylneuraminic acid yields no purple but only a pale-yellow colour with the reagent after this treatment. To these tubes, cooled, and to the others, unheated, 1 ml. of orcinol reagent was added and the tubes were kept at 100° for 15 min. The tubes were then brought to room temperature in running water, 4 ml. of pentan-1-ol was added and the tubes were stoppered, shaken and centrifuged at 2000*g* for 3 min. Solution from the upper layers was taken by Pasteur pipette into 1 cm. cells of a Unicam SP. 500 spectrophotometer and *E* at 570 mμ measured. The extinctions, less their appropriate controls, were expressed as *N*-acetylneuraminic acid by comparison with a standard curve based on the values obtained by treating in the same way samples of 10–50 μg. of this substance.

(ii) Thiobarbituric acid reaction. A few modifications have been made in the method of Warren (1959), although the periodate, arsenite and thiobarbituric acid reagents which he describes were used.

The $CHCl_3$–methanol tissue extract was evaporated to dryness in N_2 (as described above) and the residue shaken with 1 ml. of water. Of this extract 0·18 ml. was pipetted into a 125 mm. × 16 mm. stoppered tube, 0·02 ml. of N-H_2SO_4 added and the tube with others placed in a bath at 80 ± 1° for 2 hr. To the cooled tubes 0·1 ml. of periodate reagent was added and, after 20 min. at room temperature, 1 ml. of arsenite reagent was added and the tubes were shaken; a yellow–brown colour formed transitorily and disappeared. Thiobarbituric acid reagent (3 ml.) was then added and the tubes were shaken and kept in a vigorously boiling-water bath for 15 min. They were cooled in running water for 5 min., 4·3 ml. of *cyclo*hexanone was added, the whole shaken, the mixture centrifuged, the upper layer transferred to the cell of the spectrophotometer and *E* measured at 549 mμ.

A study (D. A. Booth, in preparation; some experiments with Dr L. S. Wolfe) of the course of liberation and destruction of *N*-acetylneuraminic acid during treatment of ganglioside preparations with 0·1 N-H_2SO_4 indicated 2 hr. at 80° to give maximum values, which were, however, only 61·4 ± 0·6% of the *N*-acetylneuraminic acid of the specimen. This was found to apply also to the gangliosides of tissue specimens and values reported in this paper have been corrected for the incomplete recovery; comparative values by the orcinol and the thiobarbiturate methods are given in Table 4.

Materials

The ganglioside, clupein, salmine and *N*-acetylneuraminic acid specimens were those described by McIlwain (1961*a*). Suramin, the sodium salt of bis(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamine-4:6:8-trisulphonate)carbamide, was obtained from Imperial Chemical Industries Pharmaceutical Division and from Bayer Farbenfabrik A.G. Sodium iodate, $FeCl_3$ and Na_2SO_4 were analytical reagents and pentan-1-ol, orcinol, thiobarbituric acid, sodium arsenite and *cyclo*hexanone were laboratory reagents.

RESULTS

Change in gangliosides with loss of excitability

Though values for cerebral-ganglioside content are quoted, for example by Klenk (1955) and by Svennerholm (1957), no investigation appears to have been made of whether the substances are stable during the few minutes occupied in obtaining samples from the brain of experimental animals at ordinary temperatures. As several other substances undergo rapid changes during such sampling but can be estimated after the brain has been frozen *in situ* by dropping small animals into liquid nitrogen (see McIlwain, 1959), this procedure was applied to obtain samples for determination of gangliosides. Table 1 shows that tissue so fixed yielded almost the same quantity of gangliosides,

Table 1. *Ganglioside-like substances of tissues fixed* in situ *and kept* in vitro

Tissue extraction was by procedure *A* and determination by Bial's reaction (see Experimental section); mean values are followed by S.D. and number of determinations.

Tissue	Treatment before extraction	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue)
(1) Young rats,* whole brain	Frozen *in situ*	1·83±0·16 (6)
	Removed at room temperature	1·73±0·09 (4)
(2) Adult guinea pigs, cerebral cortex	Removed at room temperature	1·87±0·08 (10)
(3) As (2)	As (2), incubated for 40 min. in glucose–glycylglycine medium	1·72±0·08 (6)
(4) As (2)	As (3), salt soln. with 150 μg. of clupein sulphate/ml.	1·35±0·09 (6)
(5)† As (2)	Left at 0° in glucose–phosphate medium for 18 hr. and subsequently incubated for 40 min. in same medium	1·40±0·09 (6)
(6)† As (2)	As (5), but for 5 hr. at 0° in tris–glucose medium before incubation	1·53±0·05 (6)

* Recently weaned and weighing 70–90 g.
† In these experiments the media also were examined for ganglioside-like extractives, as described in the text.

determined according to Long & Staples (1959), as tissue removed at room temperature: values under the two conditions did not differ significantly. The cerebral cortex from adult guinea pigs gave ganglioside values similar to those of the whole brain of young rats.

Tissue samples 3–6 of Table 1 were prepared for incubation in a uniform fashion (see Experimental section), which involved weighing them after contact with aqueous media; absorption of fluid occurs and accounts for tissues 3 giving lower ganglioside values than tissues 2. Greater changes were, however, caused by exposing the tissues to the conditions refered to in the introduction, which caused loss of their excitability. Thus the presence of protamine during incubation, or prior exposure of the tissues in cold media, caused diminution of 12–22% in the ganglioside extracted (tissues 4–6, Table 1). In two of these experiments, attempts were made to detect gangliosides in the media in which the tissues had been exposed or incubated. Although gangliosides added to such media could be estimated with an accuracy equivalent to the detection of 7 μm-moles of *N*-acetylneuraminic acid/100 mg. of tissue, none could be detected in the media when 35 μm-moles/100 mg. had been lost from the tissue.

Media in which cerebral slices had been incubated with protamine were examined semi-quantitatively for the presence of protamine by adding picric acid or fluorescein as described by McIlwain (1961*a*). These reagents form insoluble salts with protamine in the concentrations added before incubation, and showed an apparent loss of protamine from the incubating fluid. It was therefore thought that a protamine–ganglioside complex might have formed in the tissue itself, for basic proteins had proved to become attached to ganglioside-rich subcellular fractions of ground cerebral tissues (Wolfe & McIlwain, 1961). The attachment of protamine to slices has since been demonstrated quantitatively (Thomson & McIlwain, 1961). Presumably, therefore, this attachment was associated with either a breakdown of cerebral gangliosides, or with diminution in their extractability.

Binding of ganglioside-like substances in cerebral tissues; a new reagent for their extraction

Reagents which combined with basic proteins were therefore examined as aids to the extraction of ganglioside-like substances. The extraction of Folch *et al.* (1957) and Long & Staples (1959) involves grinding or blending tissues with chloroform–methanol (2:1, v/v). In testing the effect of added substances, these were dissolved or suspended in a small volume of water and the tissue was ground first with the added reagents; the mixture was then treated as usual with chloroform–methanol. Fluorescein, referred to above, was found unsuitable as a reagent, for it interfered with the subsequent determination of gangliosides. A number of acidic substances of relatively high molecular weight were being examined for their ability to restore tissue excitability (McIlwain, 1960*a*, 1961*a* and unpublished work) and several of these substances were tested as possible agents in release of gangliosides in normal and in protamine-treated tissues (Table 2).

In normal tissues freshly removed from guinea pigs, less ganglioside was obtained in the presence of polygalacturonic acid or of hydrochloric acid. Chondroitin sulphate and trypan red, though forming precipitates with protamine, did not in-

Table 2. *Acidic compounds and extraction of ganglioside-like substances*

Tissues were ground first with any added substances, and then with $CHCl_3$–methanol, as described in the Experimental section. Mean values are followed by S.D. and number of experiments, except when only two or three determinations were made.

Tissue	Added substance (quantity/100 mg. of tissue)	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue)
(1) Guinea-pig cerebral cortex removed at room temperature	None	1·74±0·08 (11)
	N-HCl, 0·1 ml.	0·81
	Polygalacturonic acid, 10 mg.	1·11±0·12 (4)
	Chondroitin sulphate, 2 mg.	1·63±0·05 (4)
	Trypan red, 2 mg.	1·71±0·11 (4)
	Suramin, 2 mg.	2·07±0·11 (4)
	Suramin, 5 mg.	2·34±0·06 (4)
	Suramin, 5 mg.*	2·36
(2) Young rats, whole brain frozen *in situ*	None	1·83±0·16 (6)
	Suramin, 1·7–2·3 mg.	2·28±0·16 (6)

* Tissue was left for 30 min. after grinding with suramin; in all other cases tissue was left for 2–3 min.

crease ganglioside extraction. Increased extraction was, however, obtained with an antitrypanosomal agent, suramin: the sodium salt of bis-(*m*-amino-benzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthyl-amine-4:6:8-trisulphonate)carbamide. Suramin gave increased extraction of ganglioside-like substances from the brain fixed *in situ* with liquid nitrogen, as well as from tissues removed promptly at room temperature; 2–3 min. exposure to suramin was adequate (Table 2). The increased ganglioside readings did not appear to be artifactual: in the absence of tissue, the full procedure of extraction, solvent distribution and determination of *N*-acetylneuraminic acid could be carried through without the appearance of 'gangliosides' if none had been added, and if they, but no tissue, had been added the same recovery (95±3%) was obtained with or without suramin. The quantity of suramin needed was concluded from the experiments of Fig. 1 to be above 3 mg./100 mg. of tissue, and 5 mg./100 mg. was used in subsequent experiments unless stated otherwise.

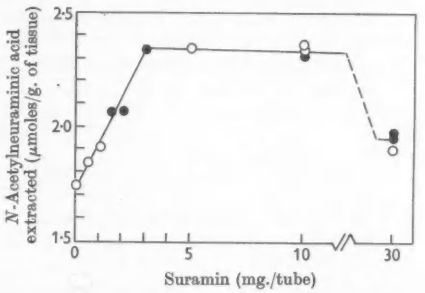

Fig. 1. Ganglioside-like material extracted from guinea-pig cerebral cortex in the presence of different quantities of suramin. Samples of 80–100 mg. of cortex were ground with suramin and $CHCl_3$–methanol as described in the Experimental section; determination was by Bial's reaction. ○ and ●, Different samples of suramin.

Recovery of added gangliosides and properties of the additional suramin-extracted material. Recovery of gangliosides added to mixtures containing cerebral tissues immediately before their extraction has now been re-appraised. Table 3 (Expts. 1 and 2) shows that under the conditions of Long & Staples (1959) recovery, though good, was significantly below 100%. In these experiments the quantity of added gangliosides was commensurate with the quantity native to the tissue. With added suramin, not only were higher values obtained for tissue gangliosides but, also, the recovery of added material was increased.

To learn more about the additional material extracted in the presence of suramin, assays for *N*-acetylneuraminic acid were made not only with Bial's reagent, which formed the basis of the assays already discussed, but also with a thiobarbiturate method based on that of Warren (1959). An examination of the thiobarbiturate method specifically in relation to gangliosides, and in comparison with Bial's reagent, is being made (see Experimental section). Table 4 shows that both methods give a similar picture of increased extraction in the presence of suramin, and both were applied to see whether *N*-acetylneuraminic acid-containing material, not extracted by chloroform–methanol alone, could subsequently be obtained by adding suramin to the tissue residue, grinding and re-extracting. Any further extraction was small. As the brain is reported to contain protein-bound *N*-acetylneuraminic acid (Svennerholm, 1956), a preliminary examination has been made by paper electrophoresis (D. A. Booth, unpublished work) to detect such substances in the present extracts. Any additional amino acid-

Table 3. *Recovery of gangliosides added to tissue*

The quantities of gangliosides noted, in 50 or 100 μl. of water, were added to one of a duplicate set of tubes; those of Expt. 3 also contained suramin (2 mg.). Weighed portions of guinea-pig cerebral cortex (approx. 80 mg.) were then added to all tubes, followed by $CHCl_3$–methanol (2:1, v/v). After grinding, the tubes were left for 15 min. and the determinations completed by Bial's reaction as described in the Experimental section.

Expt. no.	*N*-Acetylneuraminic acid (μmole/tube) Added	Found	Calc. from controls, as present in the tissue taken	Recovery (% of that added as ganglioside)
1	0·084	0·233	0·155	93±7 (4)
2	0·168	0·294	0·139	92±5 (4)
3	0·084	0·275	0·188	103±6 (4)

Table 4. *Comparison of two ways of applying suramin; two assay methods*

Blocks of about 1 g. from guinea-pig cerebral hemispheres were placed in tubes, some of which (Expt. 1) contained 20 mg. of suramin in 0·2 ml. of water. The tubes of Expt. 2 (*a*) received $CHCl_3$–methanol (2:1, v/v), 19 times the volume of the tissue, and those of Expt. 1, $CHCl_3$–methanol 19 times the volume of tissue plus added water. After grinding and centrifuging, determinations were completed as described in the Experimental section. In addition, the residue from the extraction of Expt. 2 (*a*) was ground with suramin (20 mg. in 0·2 ml. of water) and the mixture ground with $CHCl_3$–methanol as in Expt. 1.

Expt. no.	Extraction	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue) Bial's reaction	Thiobarbituric acid method
1	Suramin present initially	2·22	2·20
2	(*a*) Initial extraction without suramin	1·86	1·84
	(*b*) Residue re-extracted in the presence of suramin	No detected	0·19

containing material in extracts made in the presence of suramin was not, however, associated with *N*-acetylneuraminic acid.

Gangliosides during loss and recovery of tissue excitability

The change in gangliosides previously observed to accompany loss of excitability has now been re-investigated with the new extraction method. In Table 5, tissue treated according to procedures 3, 4 and 5 lost excitability and all are now seen to yield less gangliosides in the presence as well as in the absence of suramin. Comparison of tissues incubated without and with protamine is particularly instructive, as these are brief procedures; a proportion of the gangliosides has, however, become inextractable.

The tissue content of ganglioside-like substances has been determined also under conditions which led to recovery of excitability. After keeping in cold media, tissues were incubated with added gangliosides (Table 6). This led to increase in their ganglioside content, whereas tissues which had not been previously treated in the cold picked up much less gangliosides on incubation. In appraising these values, it is necessary to consider how much of the added gangliosides could have become associated with the tissue in extracellular fluids. At the end of the experiments slices were drained from excess of fluid but could have up to 80 mg. of extracellular fluid/100 mg. original fresh wt. of tissue. Supposing this to be completely accessible to the ganglioside solution containing 0·5 mg./3·5 ml., or 0·12 μmole of *N*-acetylneuraminic acid/ml., the associated ganglioside could contribute some 0·1 μmole of *N*-acetylneuraminic acid/g. of tissue. Thus the small increase in ganglioside content in the untreated tissue of Table 6 could be attached in this relatively mechanical fashion. After keeping at 0°, however, the tissue takes up six times this quantity of ganglioside. Treatment of the tissue with protamine also increased its ability to take up ganglioside. Suramin released additional ganglioside-like material from all tissues.

The various associations observed between tissue gangliosides and excitability led to examination of whether electrical pulses themselves changed the tissue content of ganglioside-like substances (Table 5, Expts. 6–8). No change was found, however, in the presence or absence of protamine.

Table 5. *Suramin-released gangliosides of incubated tissues*

Guinea-pig cerebral cortex was used as slices of 80–100 mg. and extracted without and with suramin, by procedures *A* and *B* of the Experimental section; determination was by Bial's reaction.

Treatment of tissue before extraction	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue)	
	Without suramin	With suramin
(1) None	1·74±0·08 (11)	2·34±0·06 (4)
(2) Incubated for 40 min., glycylglycine–glucose medium	1·72±0·08 (6)	2·35±0·09 (6)
(3) As (2), medium with 150 μg. of protamine SO_4/ml.	1·35±0·09 (6)	2·04±0·09 (4)
(4) Kept for 5 hr. at 0° in tris–glucose medium, then incubated at 37° for 40 min.	1·53±0·05 (6)	1·96±0·04 (6)
(5) Kept for 18 hr. at 0° in phosphate–glucose medium, then incubated at 37° for 40 min.	1·40±0·09 (6)	2·01±0·06 (6)
(6) Incubated for 80 min. in glycylglycine–glucose medium, with electrical pulses during the final 40 min.	1·86±0·07 (4)	2·31±0·06 (10)
(7) As (6), medium with 150 μg. of protamine SO_4/ml.	1·42±0·04 (4)	2·02±0·08 (4)
(8) As (6), medium with 500 μg. of protamine SO_4/ml.	1·51±0·08 (4)	2·09±0·06 (4)

Table 6. *Normal and treated tissues incubated with gangliosides*

Guinea-pig cerebral cortex, sliced, was incubated for 40 min. in the oxygenated media in manometric vessels. Gangliosides when added were at 143 μg./ml. in the incubation medium and protamine (clupein sulphate) at 200 μg./ml. Extraction of the tissue was by method *A* (no suramin) or *B* (with suramin). Values are the mean of two or three determinations except when followed by S.D. and number of determinations.

Treatment of tissue before extraction	Ganglioside-like extractives (μmoles of *N*-acetylneuraminic acid/g. of tissue)		
	No suramin	Suramin (2 mg.)	Suramin (5 mg.)
(1) Incubated, glycylglycine medium	1·84	2·07	2·35±0·06 (6)
(2) As (1), medium with gangliosides added after 15 min.	1·93	2·17	—
(3) Incubated, glycylglycine medium with protamine	1·42	2·04	—
(4) As (3), medium with protamine and gangliosides*	1·59±0·03 (4)	2·13±0·09 (4)	2·29±0·10 (4)
(5) At 0°, 18 hr. in phosphate medium, then incubated	1·31	1·94	—
(6) As (5), gangliosides added before incubation	1·89±0·08 (4)	2·13±0·08 (4)	2·27±0·07 (4)

* In one set of experiments, gangliosides were added from a side arm after incubation for 15 min., as in treatment (2). In another set, the protamine-containing medium was removed after 15 min. and replaced by fresh medium containing gangliosides and no protamine. The values were closely similar.

DISCUSSION

The evidence presented above gives further reasons for considering gangliosides to be involved in the electrical excitability of cerebral tissues. Previous studies (see introduction) showed their potency in restoring excitability to tissues (i) kept in cold media, and (ii) treated with basic proteins. Now (iii) the tissues kept in cold media have been found to yield less gangliosides to chloroform–methanol, as also (iv) did those treated with basic proteins. Also (v) addition of ganglioside to tissues kept in cold media led to an increase in their ganglioside content which did not occur with normal tissues; and (vi) suramin, which increased the response of tissues treated as in (i) and (ii), permitted the extraction from such tissues by chloroform–methanol of a further quantity of ganglioside-like material. The connexion of gangliosides with processes of excitation is, however, of a different type from those exhibited by, for example, glucose, which is consumed, and phosphocreatine, which undergoes a reversible change in quantity, during a few seconds' stimulation. Change was not detected in gangliosides during removal of the brain at room temperature, nor during electrical excitation *in vitro*.

Accepting the conclusion (Thomson & McIlwain, 1961) that interactions between protamine, suramin and tissue gangliosides show neuraminic acid of gangliosides to contribute negative surface groups concerned in tissue excitation, it would now appear that not all neuraminic acid residues of ganglioside-like substances are in this position. Not all such substances were rendered inextractable by protamine, and suramin released a further quantity

from normal, excitable, tissue. Investigation of whether this behaviour is due to chemical heterogeneity in gangliosides or to differences in their location in the tissue, or to both factors, is in progress. The ready combination of suramin with proteins (Town, Wills, Wilson & Wormall, 1950) suggests that the gangliosides released by suramin may be normally linked in some distinctive fashion to tissue protein. The subcellular fractions of cerebral cortex which carry gangliosides (Wolfe & McIlwain, 1961; Wolfe, 1961) carry also an adenosine triphosphatase which is activated by Na^+ and K^+ ions (J. Järnefelt, personal communication; D. H. Deul & H. McIlwain, in preparation). This enzyme is inhibited by suramin (D. H. Deul & H. McIlwain, in preparation). Gangliosides restore active Na^+ and K^+ ion transport to tissues in which this has been inhibited by protamine (Cummins & McIlwain, 1961; Woodman & McIlwain, 1961). These, with the present findings, have been incorporated into a mechanism of ion transport which is outlined by McIlwain (1960*c*).

SUMMARY

1. *N*-Acylneuraminic acid derivatives have been determined by the orcinol–ferric chloride reaction in cerebral tissues, after extraction and solvent partition by methods which yield ganglioside preparations. The resulting values for ganglioside-like substances are expressed by reference to synthetic *N*-acetylneuraminic acid. Tissues frozen *in situ* gave values of 1·8 μmoles/g., close to those of tissue dissected at room temperature.

2. Incubation in oxygenated glucose media had little effect on these values, but diminution to 1·3–1·5 μmoles/g. was found after incubating with protamine or after keeping in cold media before incubation. These are conditions under which the tissue's metabolic response to electrical pulses is lost.

3. Further quantities of ganglioside-like substances could be extracted from tissues in the presence of suramin. A number of other substances were inactive in this respect; suramin increases response by the tissue.

4. Suramin also increased the yield of ganglioside-like substances when these were estimated by an independent method which depended on hydrolysis and reaction with periodate, followed by thiobarbituric acid.

5. The total ganglioside-like substances, which included those extracted in the presence of suramin, also diminished under the conditions of paragraph 2 above. Subsequent incubation with gangliosides under conditions allowing restoration of response to pulses, increased the ganglioside extractable from the tissue. Incubation with gangliosides did not have a comparable effect on normal tissue.

6. The findings indicate heterogeneity in the tissue gangliosides and the association of at least one component with the tissue's ability to respond to pulses.

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Basic Proteins and the Potassium Movements and Phosphates of Cerebral Tissues

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An inexcitable condition in cerebral tissues is associated with migration of histones from the cell nuclei to material which appears to be derived from cell membranes (McIlwain, 1959; Wolfe & McIlwain, 1960, 1961). In ground cerebral tissues added histones and other basic proteins or polypeptides became attached to the same subcellular fractions, which were found to be rich in gangliosides (Wolfe, 1961). Tissue excitability was restored by adding gangliosides and certain related substances (McIlwain, 1960*a*, 1961*a*). Because of these indications of the chemical nature of the target or receptor for the basic proteins, knowledge of any metabolic concomitants of their action would be of value in indicating the role played by the gangliosides and possibly in revealing part of the mechanisms concerned with excitability in cerebral tissues.

Events affected by applying electrical pulses to cerebral tissues are, in likely order of immediacy: ion movements (Cummins & McIlwain, 1961), changes in tissue phosphates (McIlwain & Gore, 1951; Kratzing & Narayanaswami, 1953; Heald, 1958) and changes in respiration and glucose metabolism (McIlwain, 1951; McIlwain, Anguiano & Cheshire, 1952). Action of basic proteins on the latter group of processes has been reported (McIlwain, 1959) and action on tissue phosphocreatine, inorganic phosphate and potassium salts is now described.

EXPERIMENTAL

The conditions, except those in experiments of Table 4, were those recently described (Cummins & McIlwain, 1961) with slices of 80–100 mg. of guinea-pig cerebral cortex prepared and incubated in glucose–sodium hydrogen carbonate media equilibrated with $CO_2 + O_2$ (5:95). Slices were held in quick-transfer electrodes (Heald & McIlwain, 1956; McIlwain, 1960*b*) during incubation, whether or not electrical pulses were applied; when applied, pulses were alternating, of exponential time–voltage relationship, 10v peak potential, 0·4 msec. time-constant and at 100/sec. (see McIlwain, 1954). After incubation as described in individual experiments, slices were rapidly transferred to 100 ml. of ice-cold 0·3M-sucrose and from there to 2·5 ml. of 10% (w/v) trichloroacetic acid (Cummins & McIlwain, 1961). In the trichloroacetic acid extracts, phosphocreatine and inorganic phosphate were determined as described by Woodman & McIlwain (1961), and potassium according to Cummins & McIlwain (1961). After incubation, the potassium content of slices cut first, from the outer convexity of the cortex, is greater than that of slices cut subsequently (Cummins & McIlwain, 1961). Outer slices were included in the experiments of Table 2 but not in those of Table 3; mean potassium values are therefore higher in Table 2. The experiments in Table 4 were carried out in ordinary conical manometric vessels with the glycylglycine–glucose medium and other conditions as described by McIlwain (1959).

Protamine (clupein) sulphate was the analysed specimen of McIlwain (1961*a*); histone sulphate was prepared from calf thymus nuclei (Wolfe & McIlwain, 1961) and suramin was the sodium salt used by Thomson & McIlwain (1961).

RESULTS

Effect of protamine on phosphocreatine and inorganic phosphate

Protamine sulphate at 100 μg./ml. almost completely inhibited the respiratory response of cerebral tissues to electrical pulses (McIlwain, 1959), but in the absence of pulses this and higher concentrations were without effect on tissue phosphocreatine. This is shown in Table 1; in these experiments, slices were incubated throughout in media of the protamine content quoted. At the beginning of incubation the slices contained little phosphocreatine and their inorganic phosphate was high (McIlwain, Buchel & Cheshire, 1951). Consequently the experiments indicate that both the resynthesis and the maintenance of normal concentrations of phosphocreatine are unaffected by the protamine sulphate at 100–400 μg./ml.

Protamine, however, modified the effect of electrical pulses on tissue phosphocreatine. Pulses which normally led to loss of 70–80% of the phosphocreatine in 1–10 min., led with 150 μg. of protamine sulphate/ml. to loss of only 20–40% (Table 1). This action in protamine was nearly maximal at 100 μg./ml.; it resulted in the protamine-treated tissues containing, after pulses for 10 min., 2·5–3 times the phosphocreatine of the corresponding slices without protamine. This is a notable difference, especially as the processes which can yield phosphocreatine, namely respiration and glycolysis, are themselves limited by protamine when pulses are applied.

Inorganic phosphate of the tissues tended to increase with pulses and with protamine, but the changes were not large; reciprocal relationships between phosphocreatine and inorganic phosphate, seen during application of pulses for a few seconds only, are not necessarily to be expected in the much longer period of the present experiments.

Added agents and the potassium salts of the tissue

Tissues that had been incubated in the presence of protamine contained less potassium than those incubated in its absence (Table 2). This action was small at 100 μg. of protamine sulphate/ml., and at its maximum (200–400 μg./ml.) involved only 20 % of the tissue potassium. However, Cummins & McIlwain (1961) found that the rapid changes in tissue potassium associated with excitation involved changes of only some 20–30 % in concentration of potassium in the slices and this is also seen to be so in the experiments of Table 2 (first line). At the beginning of incubation in such experiments, tissues were low in potassium content, but stable levels were regained during incubation. Consequently the results of Table 2 (first column) could imply that either a failure to regain potassium, or an increased loss, was caused by protamine.

These processes have therefore been examined separately. Table 2 (second line) shows that when pulses were applied to tissues which had been incubated with 100 μg. of protamine/ml., the change in tissue potassium followed a similar course to that in normal tissue. Reassimilation of potassium proved, however, to be markedly inhibited by protamine. Table 3 shows, firstly (Expt. 1) the rapid re-entry of potassium observed to occur in normal cerebral tissues (Cummins & McIlwain, 1961) after electrical stimulation had diminished their potassium content by about 10 μequiv./g. The re-entry is seen (Expt. 6) to be greatly inhibited by protamine, such that the change normally occurring in 1 min. is only partly accomplished after 5 min. A histone preparation also inhibited the reassimilation. In this experiment (Table 3, Expt. 5) histone was incubated with the tissue for 90 min. before applying pulses; almost normal reassimilation of potassium followed when incubation was for 30 min. only. This delay in the action of histone was noted earlier in relation to respiratory response to pulses (McIlwain, 1959) and is interpreted as related to diffusion of the histone.

Other circumstances related to re-entry of potassium. The re-entry did not occur when glucose was omitted from the ambient medium. Tissue in such media contained less potassium, but lost the usual quantity on excitation (Cummins & McIlwain, 1961); this was not restored during a 5 min. recovery period (Table 3). In the presence of 2:4-dinitrophenol, a normal content of potassium was reached during preincubation; pulses again

Table 1. *Preservation of phosphocreatine by protamine during application of pulses*

Slices of guinea-pig cerebral cortex (80–100 mg.) were incubated for 40 min. in 5 ml. of glucose–sodium hydrogen carbonate medium. Pulses when applied occupied the last 1 or 10 min. of incubation and were of 10 v peak potential, 0·4 msec. duration and at 100/sec.; they continued until the tissue was plunged into sucrose. It was then rapidly transferred to trichloroacetic acid. Mean values are followed by S.D. where appropriate, and by number of observations (in parentheses).

Protamine sulphate (μg./ml.)	Tissue content (μmoles/g.)					
	No pulses		Pulses (1 min.)		Pulses (10 min.)	
	Phospho-creatine	Inorganic phosphate	Phospho-creatine	Inorganic phosphate	Phospho-creatine	Inorganic phosphate
0	1·50±0·15 (5)	3·2±0·2 (5)	0·5 (2)	3·8 (2)	0·32±0·14 (5)	4·1±0·5 (5)
100	1·45±0·26 (5)	3·7±0·35 (5)	1·0±0·13 (4)	4·1±0·4 (4)	0·9±0·2 (4)	3·7±0·4 (4)
150	1·50 (2)	4·0 (2)	1·16 (2)	4·0 (2)	0·81 (2)	3·9 (2)
200	1·56±0·3 (4)	3·6±0·3 (4)	1·04 (2)	4·3 (2)	0·84 (2)	3·7 (2)
400	1·55 (2)	3·8 (2)	—	—	—	—

Table 2. *Loss of tissue potassium caused by pulses and by protamine*

Guinea-pig cerebral cortex was treated, and results are expressed, as described in Table 1.

Protamine sulphate (μg./ml.)	Tissue potassium (μequiv./g.)		
	No pulses	Pulses (1 min.)	Pulses (10 min.)
0	61±4 (8)	56±3 (8)	47±4 (8)
100	58±4 (6)	48±2 (4)	44·5±3 (4)
150	53·5±4·3 (4)	—	45·5±4 (4)
200	50·5±1 (4)	—	—
400	50·5 (2)	—	—

Table 3. *Loss and recovery of tissue potassium*

In Expts. 1–7, inner slices of guinea-pig cerebral cortex (80–100 mg.) were incubated in 5 ml. of glucose–sodium hydrogen carbonate medium for 30 min. (Expt. 5 only: for 90 min.), followed by 10 min. during which pulses were applied to the tissues of columns (*b*), (*c*) and (*d*). Tissues (*a*) and (*b*) were transferred to sucrose at this point; incubation of tissues (*c*) and (*d*) continued as indicated. Transfer and other details: see Table 1. In Expts. 8–10, incubation was as just described, but for 15 min., and was followed by transfer to a second batch of medium, already oxygenated and at 37°, in which the tissue remained for 15 min. before application of pulses and transfer to sucrose as described above.

Group of Expts.	Change in medium	Tissue potassium (μequiv./g.) (*a*) No pulses	(*b*) Pulses	(*c*) Pulses and 1 min. recovery	(*d*) Pulses and 5 min. recovery
1	None	52±3 (23)	40±3 (18)	47±2 (5)	50 (2)
2	No glucose	39±3 (4)	26±3 (4)	29 (2)	24 (2)
3	30 μM-2:4-Dinitrophenol	51±4 (5)	41±3 (5)	42 (2)	42 (2)
4	Ganglioside, 0·3 mg./ml.	54±3 (4)	44±3 (7)	49±2 (7)	52 (3)
5	Histone sulphate, 0·1 mg./ml.	54±4 (4)	44±1 (4)	46±3 (4)	46 (2)
6	Protamine sulphate, 0·1 mg./ml.	46±4 (14)	40±4 (7)	39±2 (5)	43±2 (7)
7	Protamine sulphate, 0·1 mg./ml., and ganglioside, 0·3 mg./ml.	53±1 (4)	42±4 (4)	50±3 (4)	51±1 (4)
8	First medium: as 6; second: as 1	53 (2)	44 (2)	47±1 (4)	46±3 (4)
9	First medium: as 6; second: as 4	52 (2)	43 (2)	49 (3)	50 (2)
10	First medium: as 6; second, with suramin, 1 mg./ml.	54 (2)	44 (3)	51±3 (4)	51 (3)

Table 4. *Protamine and the stimulated respiration of cerebral tissues*

Manometric vessels contained slices of guinea-pig cerebral cortex of about 60 mg. in 3·5 ml. of glycylglycine–glucose medium in oxygen at 37·5°. Protamine when added was present from the beginning of the experiment. Respiratory rate was measured during 30–40 min. shaking, and then medium or mM-2:4-dinitrophenol (DNP) in medium or M-KCl was tipped from side arms to give the concentrations indicated. Measurement of respiration was continued for a further 30–40 min. Rates quoted are mean values of the number of observations in parentheses, ±S.D. when appropriate.

Protamine sulphate of media (mg./ml.)	Additions (concn. of added substances) None	DNP (30 μM)	DNP (75 μM)	KCl (30 mM)	KCl (60 mM)
	Respiratory rate (μmoles of O_2/g. of tissue/hr.)				
0	63±2 (5)	137 (2)	127 (3)	120 (2)	116 (2)
0·1	63±3 (4)	122 (2)	134±6 (4)	75 (2)	81 (2)
0·3	61 (2)	124 (2)	134 (2)	61 (2)	69 (2)

caused loss, but no recovery occurred when pulses were stopped. The concentration of the nitrophenol was chosen as the least needed for a marked effect on respiration and phosphates of cerebral tissues (McIlwain & Gore, 1951). The increase in respiration caused by the dinitrophenol is shown in Table 4 and is not inhibited by protamine, indicating that protamine is not acting directly on respiratory processes; protamine did, however, inhibit the increase in respiration caused by 30–60 mM-potassium chloride, as well as that caused by electrical pulses (McIlwain, 1959).

If after incubation with protamine, tissues were removed from the protamine-containing medium to a normal protamine-free medium (Table 3, Expt. 8), loss of potassium with pulses was readily observed but little re-entry took place after pulses were stopped. This is consistent with the previously observed attachment of protamine to tissues (Thomson & McIlwain, 1961) and its continued inhibition of respiratory response to pulses (McIlwain, 1959).

Gangliosides and suramin. Loss and recovery of potassium were normal in tissues incubated in the presence of gangliosides (Table 3, Expt. 4; these used a ganglioside concentration about that normal to the tissue, and capable of restoring respiratory response to tissues pretreated with protamine: McIlwain, 1959). When tissues were incubated in media containing an inhibitory concentration of protamine together with gangliosides, movements of potassium were again normal (Table 3, Expt. 7). In this situation less protamine becomes attached to the tissue (Thomson & McIlwain, 1961). More significant in appraising the action of gangliosides are Expts. 8–9 of Table 3. Here tissues were preincubated in protamine-containing media and transferred to protamine-free media, some of which contained gangliosides. In this situation protamine remains attached to the tissue (Thomson & McIlwain, 1961); nevertheless, normal recovery of potassium salts occurred. Suramin acted as did gangliosides (Table 3, Expt. 10).

DISCUSSION

Cerebral tissues incubated under the present conditions regain potassium salts from the surrounding medium against a concentration gradient and with the expenditure of metabolically derived energy, as is described quantitatively by McIlwain (1960*c*) and Cummins & McIlwain (1961); see also Krebs, Eggleston & Terner (1951). The energy requirement is indicated in the present experiments by the lack of potassium re-entry when media lacked glucose or contained 2:4-dinitrophenol and glucose; tissues incubated under either of these conditions contain little or no phosphocreatine (McIlwain & Gore, 1951; McIlwain, 1952) and diminished adenosine triphosphate (Kratzing & Narayanaswami, 1953). Lack of glucose had the more drastic effect on potassium assimilation, for it yielded tissue low in potassium even in the absence of pulses. Presumably 2:4-dinitrophenol allows the formation of energy-rich phosphate ($\sim$P) by glycolysis, and glycolysis increases in its presence until some 100–150 μmoles of lactate are formed/g. of tissue/hr. (McIlwain & Gore, 1951). Increase in tissue potassium after pulses normally proceeds at some 600 μequiv./g. of tissue/hr., and with a $\sim$P/potassium ratio of about 1 (Cummins & McIlwain, 1961); it is thus understandable that it failed in the presence of the dinitrophenol. The rate of increase in the potassium content of normal tissues during the present experiments contrasts with that noted in an accompanying paper (Woodman & McIlwain, 1961) to follow the addition of glutamate, this rate being about 20 μequiv./g. of tissue/hr. and consequently unlikely to make much contribution to the recovery after pulses.

The increase in potassium after pulses represents the utilization of a considerable proportion of the tissue's metabolically derived energy (Cummins & McIlwain, 1961) and is the process now seen to be inhibited by protamine. Moreover, protamine in inhibiting potassium reassimilation did not diminish phosphocreatine, as did 2:4-dinitrophenol or the absence of glucose: by contrast, protamine preserved phosphocreatine from the diminution usually caused by pulses. Protamine can thus be pictured to be acting on a system which utilizes energy-rich phosphate in ion transport, and this system as operating in tissue as ordinarily incubated, though at a slower rate than after pulses. Thus the lower potassium of the protamine-treated tissues of Table 2 could represent an action on the same process as displayed in Table 3, though only in the latter case are pulses applied.

Restoration of potassium reassimilation by gangliosides confirms previous conclusions that they can play a role in ion transport (Balakrishnan & McIlwain, 1961; see also Bogoch, 1960). In considering this role of gangliosides in relation to their chemical nature, it is noteworthy that they are strongly ionized carboxylic acids of equivalent weight and minimum molecular weight about 1,500 (McIlwain, 1961*b*); they form firm associations with other cerebral constituents (van Heyningen, 1959), and in ground cerebral tissues they occur in membrane-derived fractions (Wolfe, 1961). These properties support a picture of ion transport in which the gangliosides are membrane constituents but are not mobile carriers, constituting rather a hydrophilic path for cation movement through the lipid membrane. A mechanism has been proposed (McIlwain, 1960*c*) in which gangliosides function at membrane pores of the type envisaged by Danielli (1958), Harris (1960) or Mullins (1959) in association with the sodium- and potassium-activated adenosine triphosphatase present in neural (Skou, 1957, 1960) and cerebral tissues (Hess & Pope, 1957; J. Järnefelt, personal communication; D. H. Deul & H. McIlwain, in preparation.) Protamine could inhibit cation transport in such a pore by combining with acidic groups at its mouth, so interrupting a sequence of negatively charged adsorption sites; and the polyacidic suramin or added gangliosides could restore an acidic environment by masking the basic groups of the protamine while still leaving it attached to the membrane.

SUMMARY

1. Guinea-pig cerebral cortex was maintained *in vitro* and its content of potassium salts and phosphocreatine measured under normal conditions, immediately after applying electrical pulses and during subsequent recovery.

2. Protamine and histone sulphates (0·1 mg./ml.) did not affect the loss of potassium induced by pulses, but inhibited recovery of potassium. This inhibition was removed by gangliosides and by suramin. Higher concentrations of protamine diminished tissue potassium in the absence of pulses.

3. Protamine (0·1–0·4 mg./ml.) was without effect on phosphocreatine in the absence of pulses, but in their presence it preserved a large part of the phosphocreatine from the loss normally caused by pulses.

4. Protamine and histone are considered to act at a mechanism which utilizes a major part of the energy-rich phosphate of the tissue in ion transport, and suggestions are made about the nature of this mechanism.

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Glutamic Acid, other Amino Acids and Related Compounds as Substrates for Cerebral Tissues: Their Effects on Tissue Phosphates

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This investigation was prompted by the apparently opposed actions shown by glutamic acid in cell-free and in cell-containing systems derived from mammalian brain.

When cell-containing cerebral tissues respire in glucose media, phosphocreatine is resynthesized from creatine already present in the tissue; addition of glutamic acid then increases respiratory rate (Weil-Malherbe, 1936) but decreases phosphocreatine (McIlwain, Buchel & Cheshire, 1951; McIlwain, 1952). When added as only oxidizable substrate, glutamic acid again supports relatively high respiratory rates but does not lead to resynthesis of phosphocreatine. Nevertheless, with ground cerebral tissues or with mitochondrial preparations from them, glutamic acid supports oxidative phosphorylation (Brody & Bain, 1952), and thus would be expected to lead to the accumulation of energy-rich phosphates including phosphocreatine.

One interpretation of these findings is that they indicate the occurrence of additional, energy-consuming processes in the cell-containing tissue, processes which are diminished with loss of cell structure or not represented in the mitochondrial systems. Thus when it was observed that the addition of glutamic acid to tissues respiring in glucose increased the quantity of potassium salts associated with a given weight of tissue (Terner, Eggleston & Krebs, 1950), the assimilation of potassium salts appeared likely to represent the additional energy-consuming process. Pappius & Elliott (1956) have since shown that the additional potassium under these conditions is associated with increased volume, the concentration of potassium salts falling or remaining little changed.

In the present study, other processes which might lead to loss of adenosine triphosphate or phosphocreatine in the presence of glutamate have been examined. These include the synthesis of glutamine, noted by Krebs (1935) and Weil-Malherbe (1936), the appearance of inorganic pyrophosphate from reactions such as those described with amino acids by Moldave, Castelfranco & Meister (1959) and the displacement of creatine from the tissue. In addition, evidence has been sought by comparing the action of glutamic acid on tissue phosphates with the actions of other amino acids and with related keto and guanido derivatives.

EXPERIMENTAL

Tissues and media

Three different preparations of tissues were made from the brains of young and adult rats, and of guinea pigs.

(*a*) For tissue frozen *in situ*, young rats of 50–100 g. were dropped into liquid N_2 in a vacuum flask and the brain of each was chiselled out with cold tools as described by Richter & Dawson (1948) and McIlwain *et al.* (1951). (*b*) Tissue slices, unless otherwise stated, were prepared from adult guinea pigs, only the cerebral cortex (and not subcortical white matter), removed from the animal within 90 sec. of death, being used. Three slices were cut successively from each hemisphere with a blade and guide (McIlwain, 1961) lubricated with the medium subsequently used for incubation. Values for the slices cut at different depths in the cortex are given separately only in Table 1. After cutting, the slices were floated from the blade or guide into a shallow dish of medium and trimmed to a chosen weight, usually 80–100 mg. (*c*) For experiments of Table 6 only, slices were cut with a blade and guide not lubricated with medium, and were picked from the guide with a bent wire rider. (*d*) Ground cerebral tissues were prepared from blocks of cerebral cortex which included both grey and white matter. About 0·5 g., weighed on a torsion balance, was added to 5 ml. of 0·9% NaCl at 0° in a test-tube homogenizer and ground for 15 sec.

The inorganic constituents of the fluid media used with tissue slices were based on previous practice except that phosphates were not used as buffers because to do so involved phosphate concentrations greater than those that exist *in vivo*, and the present studies concerned tissue phosphates. The glycylglycine medium contained (mM): NaCl, 128; KCl, 5; KH_2PO_4 and $MgSO_4$, 1·3; $CaCl_2$, 2·8; glycylglycine, brought to pH 7·4 by NaOH, 30. Glutamic acid affects similarly the phosphates of tissues incubated in $NaHCO_3$-buffered and glycylglycine-buffered media (McIlwain, 1952), and to examine concomitant effects on respiration glycylglycine buffer has been used in the present experiments. Glucose was normally employed as substrate at 10 mM, and was present throughout the experiment unless stated otherwise.

L-Aspartic acid, L-asparagine, L-leucine, L-proline, L-hydroxyproline, L-lysine hydrochloride, L-histidine hydrochloride, L-tryptophan, L-tyrosine, D-glutamic acid and α-oxoglutaric acid were from L. Light and Co. Ltd.; L-alanine, β-alanine, L-isoleucine, L-methionine, L-threonine, L-serine, L-valine and L-glutamine were from British Drug Houses Ltd.; L-citrulline and L-phenylalanine were from Roche Products Ltd., L-glutamic acid and L-ornithine from Merck Corp. (U.S.A.), oxaloacetic acid from the Biochemical Corp. (Calif., U.S.A.) and sodium pyruvate from C. F. Boehringer und Soehne. γ-Aminobutyric acid, chromatographically pure, was kindly given by Dr D. B. Tower, National Institute of Neurological Diseases and Blindness, U.S. Public Health Service, and γ-guanidobutyric acid was prepared from it with cyanamide as Dubnoff (1957) described for the preparation of glycocyamine. The product melted at 182° and was identical chromatographically with an authentic specimen given by Dr I. Smith, Courtauld Institute of Biochemistry.

Analytical methods

Oxygen uptake was measured manometrically by conventional methods. Unless stated otherwise, slices of 80–100 mg. were employed in 3·5 ml. of fluid in a vessel of 15–20 ml. In a few instances slices totalling 300–500 mg. in weight were examined in 15 ml. of fluid in vessels of 100 ml. For analysis of tissue constituents the tissue specimen was removed promptly from the fluid at the end of the experiments with a mounted bent wire and in different instances certain of the determinations of the following paragraphs were carried out. Lactic acid formation was measured in the fluid by the method of Barker & Summerson (1941).

Weight. Slices were drained from fluid by repeated contact with a glass surface, while held on the wire rider of a torsion balance. They were weighed wet and then placed in small tared crucibles and dried to constant weight at 100° (1·5 hr.).

Orthophosphate and phosphocreatine in slices. The tissues were collected with a mounted bent wire, drained for 2 sec. only on the side of the vessels from which they were being removed, and placed in 1·5 ml. of 0·6 M-trichloroacetic acid, ice-cold in a centrifuge tube. They were immediately ground in the acid with a homogenizer pestle which fitted the tube, and the pestle was washed with 1 ml. of the trichloroacetic acid. After centrifuging, 2 ml. of supernatant was taken to another centrifuge tube for determinations based on the methods of Berenblum & Chain (1938), Long (1943) and Heald (1954). The supernatants from a number of slices, together with appropriate standards, in separate tubes, were brought to pH 8·4 at 0° with 5 N-NaOH (about 0·25 ml.), 0·1 ml. of 0·1 M-$NaHCO_3$ and 0·25 ml. of 25% (w/v) barium acetate were added, and the tube was shaken and after 20 min. centrifuged. The supernatant (i) was transferred to a further tube, the residue suspended in 1 ml. of a solution 50 mM in both barium acetate and NaOH, centrifuged, and this supernatant (ii) added to supernatant (i).

For orthophosphate determination, the washed residue was dissolved in 1 ml. of 0·2 N-HCl, the solution made to 4 ml. with water and 0·5 ml. of 10 N-H_2SO_4 added. Ammonium molybdate [1 ml. of 5% soln. (w/v; 40 mM)] was added, the mixture shaken, 4 ml. of *iso*butanol added and the tubes were stoppered and shaken vigorously for 15 sec. The aqueous layer was removed with a Pasteur pipette and the *iso*butanol layer was shaken with 2 ml. of N-H_2SO_4; this was also removed, and 2 ml. of 35 mM-stannous chloride in N-H_2SO_4 was added. The tubes were shaken, the aqueous layer was removed, the *iso*butanol was made to 10 ml. with ethanol and shaken and centrifuged to remove traces of $BaSO_4$, and E was measured at 700 mμ.

For phosphocreatine, to the supernatants (i) and (ii) (combined volume 3·5 ml.) were added 19 ml. of ethanol and 0·1 ml. of 0·1 N-$NaHCO_3$, the tubes were shaken, left at 0° for 1 hr. and centrifuged. The supernatant was removed, the residue was dissolved in 0·2 N-HCl and treated as described in the preceding paragraph, except that after addition of the ammonium molybdate the tubes were incubated at 30° for 30 min. before *iso*butanol extraction. This residue contains a small proportion of the tissue's adenosine triphosphate, which does not, however, yield inorganic phosphate under these conditions (Ennor & Stocken, 1948).

To find whether the alkaline conditions of the phosphate separation just described caused change in tissue phos

phates, the following determinations, which avoid alkali, were also carried out. For inorganic phosphate the method of Lowry & Lopez (1946), with 0·2 mM-$CuSO_4$ as indicated by Peel, Fox & Elsden (1955), was applied to samples of the trichloroacetic acid extracts of slices, prepared as described above. Phosphocreatine plus inorganic phosphate was determined in further samples by exposing them, after molybdate addition, to 30° for 30 min. and then completing the determination as described in the preceding section.

Phosphates in media and reaction mixtures. For inorganic phosphate of media from which slices had been removed, 0·2–3 ml. was taken according to the expected phosphate content, brought to 5 ml. and made N in H_2SO_4 by addition of water and 10N-H_2SO_4; molybdate was added and the determination completed as described above.

In reaction mixtures containing ground tissue and added phosphocreatine, precipitation of a salt of phosphocreatine was not necessary. The mixture (0·5 ml.) was added to 1·5 ml. of trichloroacetic acid at 0°, centrifuged and 0·5 ml. of the supernatant was taken and inorganic phosphate precipitated as described above. The supernatant was then treated directly with H_2SO_4 and molybdate at 30° for 30 min., to determine phosphocreatine.

Creatine. The free creatine of tissues was determined by transferring them immediately after incubation to 1·5 ml. of 5% (w/v) sulphosalicylic acid in a centrifuge tube, grinding with a pestle and centrifuging. In portions of the supernatant the diacetyl method was applied as described by Thomas (1956).

Inorganic pyrophosphate. Method (i). The following method is based on that of Flynn, Jones & Lipmann (1955). Tissue (about 100 mg.) was ground in 2·5 ml. of 10% (w/v) trichloroacetic acid, and, after centrifuging, 2 ml. of the supernatant was adjusted to pH 7 with 5N-NaOH. Barium acetate (0·25 ml. of 25% soln., w/v) and 0·1 ml. of 0·1N-$NaHCO_3$ were added and after 30 min. at 0° the precipitate was collected by centrifuging and washed with 1 ml. of 50 mM-barium acetate, pH 8·4. It was then suspended in 4 ml. of water, 0·1N-HCl was added to give a solution and the pH brought to 7. Exactly 0·5 ml. of 10N-H_2SO_4 and 1 ml. of 2·5% ammonium molybdate were added, and the mixture was shaken with *iso*butanol. The lower layer was washed twice with 2 ml. of benzene, 0·1 ml. of 10 mM-$CuSO_4$ was added, followed by the 1:2:4-aminonaphtholsulphonic acid reagent (0·4 ml.; containing 0·25 g. of the acid, 7·5 g. of sodium metabisulphite and 1·5 g. of $Na_2SO_3,7H_2O$ in 100 ml.). Cysteine (1 ml. of 0·1M) was added, the mixture made to 10 ml., centrifuged and *E* at 700 mμ read 90 min. later. In this modification of the method, extraction with *iso*butanol prevents orthophosphate from interfering with the reaction and enables it to be determined separately. Washing with benzene was found necessary to remove the *iso*butanol, which yielded an extraneous colour. The copper increased *E* from a given amount of pyrophosphate, enabling 0·02 μmole to be determined. Satisfactory values and recovery of pyrophosphate were obtained from incubated cerebral tissue, but not from the quickly frozen tissue. In this situation method (ii), based on observations of Crane & Lipmann (1953) and Umbreit, Burris & Stauffer (1957), was used.

Method (ii). Tissue (about 100 mg.) was treated as in method (i) to yield the water-insoluble barium salts, and these were dissolved in 2 ml. of 0·05N-trichloroacetic acid. Norit (40 mg.; prepared from SX grade by boiling with 2N-HCl for 20 min. and washing on a Buchner funnel with water until the washings were neutral: see Hawk, Oser & Summerson, 1947) was added and the tubes were shaken for 15 min., centrifuged, the supernatant was transferred to a 10 ml. stoppered tube and the charcoal washed twice with 1 ml. of 0·2M-sodium acetate, pH 4·5. To the combined supernatant and washings 0·5 ml. of 10N-H_2SO_4 was added and the volume made to 5 ml. Ammonium molybdate (0·3 ml., 40 mM) and 4 ml. of *iso*butanol were added, the tube was shaken, the upper layer removed and the lower aqueous layer re-extracted with 2 ml. of *iso*butanol and this was again removed. The tube with the aqueous phase was then placed in a boiling-water bath for 15 min., rapidly brought to room temperature and a further 0·5 ml. of 40 mM-ammonium molybdate added. Extraction with 4 ml. of *iso*butanol now gave the phosphate derived from pyrophosphate, and this was determined after treatment with stannous chloride as described above.

Ammonia and glutamine. At chosen times during incubation, slices were rapidly removed from manometric vessels and ground in 1·5 ml. of ice-cold trichloroacetic acid (10%, w/v). The mixture was centrifuged, the supernatant collected, the residue ground with a further 1 ml. of trichloroacetic acid, the supernatants were combined and samples (1 ml.) made pH 5–6 with 10N-NaOH. Ammonia was measured in the samples in Conway units, with 1 ml. of sat. K_2CO_3 in the outer ring and 10 mM-HCl used to trap the NH_3; colorimetric estimation according to Russell (1944) was followed. For glutamine, a further sample (1 ml.) before neutralizing was placed in a water bath at 70° for 75 min. (see Harris, 1943), cooled, taken to pH 5–6 and NH_3 was determined as described above; the NH_3 formed during hydrolysis was taken as a measure of the glutamine. L-Glutamine itself was found to yield 4% of its amide N as NH_3 during the exposure to K_2CO_3; no correction has been applied for this.

RESULTS

Specificity of action of glutamic acid on phosphocreatine

Incubation of cerebral cortical slices in the standard medium with glucose and oxygen allowed resynthesis of phosphocreatine to concentrations of 1·6–1·7 μmoles/g. (Table 1). Similar values were found after periods of 30–60 min. incubation, and for investigating the effects of added substances a period of 40 min. was chosen. If glucose was not present during this period, respiratory rate, lactic acid accumulation and phosphocreatine were all lower than in its presence and inorganic phosphate was greater.

Actions of 20 amino acids. Table 2 shows, first, the previously described action of L-glutamic acid in depleting the tissue's phosphocreatine and increasing its inorganic phosphate. This property is now shown to be shared by D-glutamic acid and by L-aspartic acid. Glutamine and asparagine are markedly less active, as also are L-alanine and (of little effect) β-alanine. The many substances which are inactive at concentrations up to 29 mM include valine, leucine, isoleucine, lysine, ornithine,

Table. 1 *Water, inorganic phosphate and phosphocreatine of slices of guinea-pig cerebral cortex*

The slice cut first from the outer convexity of a cerebral hemisphere is described as an 'outer' slice and the two cut subsequently as 'inner' slices. The medium, unless stated otherwise, was glycylglycine-buffered, oxygenated and with glucose, and incubation was for 40 min. at 37°. Values for the phosphates refer to the moist tissue drained free from adhering fluid. Results are followed by S.D. and, in parentheses, the number of determinations.

Tissue preparation	Treatment before analysis	Water (%)	Inorganic phosphate (μmoles/g.)	Phosphocreatine (μmoles/g.)
Block	None	81·0±0·5 (7)	—	—
Slices*	In medium 2–4 min., room temp.	85·1±0·8 (24)	—	—
Slices*	In medium 8–10 min., room temp.	87·5±0·8 (18)	5·52±0·60 (4)	0·94±0·28 (4)
Outer slices†	Incubated in medium	89·0±0·8 (8)	3·60±0·25 (42)	1·71±0·13 (42)
Inner slices†	Incubated in medium	88·8±1·1 (16)	3·35±0·38 (66)	1·58±0·26 (68)
Inner slices‡	Incubated in medium without glucose	—	6·7±1·1 (11)	0·37±0·18 (11)

* Values for inner and outer slices did not differ significantly.
† Respiratory rate: 62±3 (40) μmoles of O_2/g./hr.; lactic acid accumulating: 38±5 (30) μmoles/g./expt.
‡ Respiratory rate: 38±5 (21) μmoles of O_2/g./hr.; lactic acid accumulating: 6·7 (3) μmoles/g./expt.

proline, hydroxyproline and threonine. There is thus a certain but fairly wide specificity in the group glutamic acids–aspartic acid–alanine in bringing about loss of phosphocreatine.

The action of L-glutamic acid on phosphocreatine is associated with increase in inorganic phosphate and respiration. These properties are associated also in L-aspartic acid, but not in the other amino acids which cause loss of phosphocreatine. Increased accumulation of lactic acid, which is also a feature of the action of L-glutamic acid, again does not always occur with these other amino acids.

Two amino acids were found to increase the phosphocreatine resynthesized by cerebral tissues: these were methionine and phenylalanine. In methionine this action was not associated with change in respiration, but in phenylalanine it was accompanied by decrease in both respiration and glycolysis. Tryptophan showed these latter effects without change in phosphocreatine but with diminution in tissue orthophosphate. A large measure of independent variation can thus take place in the four metabolic properties recorded in Table 2.

Actions of further amino, keto and guanido acids. Knowledge of metabolic routes in which glutamic acid and aspartic acid and alanine are involved in cerebral tissues led to an examination of the effects of the compounds of Table 3. Considering first the corresponding keto acids, it is seen that their actions in relation to phosphocreatine differed from those of the amino acids. Each compound, alone, brought about resynthesis of the tissue's phosphocreatinine. When present together with glucose, none lowered the phosphocreatine yielded by glucose. Actions of the keto acids on respiratory rate, however, paralleled those of the corresponding amino acids. Thus α-oxoglutarate and oxaloacetate increased respiration in the presence of glucose whereas pyruvic acid (like alanine) did not. The hydroxy acids tested, lactic acid and malic acid, supported phosphocreatine resynthesis and moderate respiratory rates.

γ-Aminobutyric acid, readily produced from glutamic acid by cerebral preparations (Roberts & Frankel, 1950, 1951), proved similar to glutamic acid in its relationship to phosphocreatine: it did not itself support resynthesis and depressed phosphocreatine resynthesis in the presence of glucose. The depression was not as great as that caused by glutamate. γ-Guanidobutyric acid, formed from the amino compound by cerebral preparations (Pisano & Udenfriend, 1958), was without effect on any of the properties measured, as also were arginine and citrulline; some depression of phosphocreatine was caused by histidine. None of these actions seemed of sufficient magnitude to contribute to understanding the action of glutamic acid on phosphocreatine.

Basis for the action of glutamic acid on phosphocreatine

(i) Glutamic acid might displace tissue creatine and thus diminish phosphocreatine. With and without added creatine, a gradient in free creatine is maintained in favour of the tissue when glucose and oxygen are available (Thomas, 1957). Results of Table 4 show, however, that glutamic acid did not decrease tissue creatine, whether or not additional creatine was provided; nor did aspartate or β-alanine.

(ii) Evidence has been given (Moldave *et al.* 1959) of interactions with adenosine triphosphate to yield enzyme-bound amino acid acylates and inorganic pyrophosphate. In the belief that such reaction might be part of a system leading to loss of energy-rich phosphate on addition of glutamate, determination of cerebral pyrophosphate was carried out. Values for the content of this

Table 2. *Metabolic effects of 20 amino acids on guinea-pig cerebral cortex respiring with glucose*

Tissues were fixed for analysis after 40 min. at 37·5°, some in the presence and some in the absence of the added compound. Values with the added compounds are quoted, followed by their difference from those of the tissues without the addition but in the same experiment. Values with added compounds may also be compared with mean values for normal tissues throughout the study, quoted in Table 1. Substances leading to no significant change in inorganic orthophosphate, phosphocreatine, respiration or glycolysis (i.e. of <0·3, 0·3, 5 or 5 units respectively) were: 20 mM-threonine, 5 and 25 mM-leucine, 20 mM-isoleucine, 20 mM-lysine, 20 mM-ornithine, 20 mM-proline, 20 mM-hydroxyproline and 20 mM-valine.

	Inorganic phosphate (μmoles/g.)		Phosphocreatine (μmoles/g.)		Respiration (μmoles of O_2/g./hr.)		Lactic acid accumulation (μmoles/g./expt.)	
Compound added (mM)	With addition	Change caused by addition	With addition	Change caused by addition	With addition	Change caused by addition	With addition	Change caused by addition
L-Glutamic acid 5	4·10±0·15 (4)	+0·25	0·48±0·05 (4)	−0·98	83±6 (5)	+24	—	—
25	4·27±0·6 (10)	+0·82	0·34±0·13 (10)	−1·11	83±7 (20)	+24	46 (2)	+11
D-Glutamic acid 5	4·47±0·3 (6)	+0·98	0·69±0·37 (6)	−1·04	60±1 (6)	0	—	—
15	4·33 (2)	+0·94	0·4 (2)	−1·35	59	2	—	—
L-Glutamine 5	2·88 (2)	−0·06	1·01 (2)	−0·01	60 (2)	−1	—	—
20	3·58±0·52 (11)	−0·09	1·11±0·3 (11)	−0·23	60±3 (10)	+2	—	—
L-Aspartic acid 7	3·56 (2)	+0·04	0·95 (2)	−0·63	—	—	—	—
20	4·35±0·3 (6)	+0·41	0·72±0·04 (6)	−0·83	75±11 (10)	+13	43	+4
36	3·65 (2)	+0·13	0·27 (2)	−1·30	—	—	—	—
L-Asparagine 5	3·44±0·3 (8)	+0·04	1·11±0·2 (8)	−0·35	63±10 (7)	+5	31 (3)	−3
20	3·63±0·2 (6)	+0·15	0·91±0·17 (6)	−0·55	63 (4)	+5	38 (3)	+2
L-Alanine 5	3·63 (2)	+0·06	0·88 (2)	−0·59	64 (2)	+2	—	—
25	3·59±0·28 (5)	+0·02	0·95±0·15 (5)	−0·52	—	—	—	—
β-Alanine 5	2·95 (2)	−0·31	1·23 (2)	−0·24	71 (2)	+3	—	—
20	3·38±0·22 (5)	−0·09	0·68±0·05 (5)	−0·79	71±3 (5)	+5	—	—
L-Serine 20	3·24±0·17 (6)	−0·43	1·29±0·17 (6)	−0·33	61±3 (6)	−1	—	—
L-Methionine 20	3·19±0·15 (5)	−0·29	1·97±0·2 (5)	+0·52	59±3 (7)	−2	—	—
L-Phenylalanine 20	3·14±0·17 (8)	−0·33	1·77±0·17 (8)	+0·38	47±3 (13)	−14	26 (3)	−9
L-Tryptophan 20	2·63 (3)	−0·73	1·53 (3)	+0·01	42±3 (6)	−17	20 (3)	−16
L-Tyrosine 20	3·4±0·17 (6)	−0·12	1·55±0·44 (6)	−0·13	46±3 (6)	−17	35 (3)	−7

Table 3. *Metabolic effects of amino, keto and guanido acids*

Tissues were incubated for 40 min. at 37·5°, during which respiration was measured. They were then fixed for determination of phosphates and samples of media taken for determination of lactic acid. +, Glucose added at 10 mM. Respiration falls to zero between 30 and 40 min. No significant changes were caused by 20 mM-citrulline, 5 or 20 mM-arginine, or 5 or 25 mM-γ-guanidobutyric acid in the presence of glucose.

Glucose	Acid added (mM)	Inorganic phosphate (μmoles/g.)	Phosphocreatine (μmoles/g.)	Respiration (μmoles of O_2/g./hr.)	Lactic acid accumulation (μmoles/g./expt.)
0	α-Oxoglutaric, 20	5·25±0·48 (7)	0·96±0·05 (7)	54±8 (14)	—
+	α-Oxoglutaric, 5	3·87 (2)	1·38 (2)	59	—
+	α-Oxoglutaric, 20	3·47±0·38 (6)	1·42±0·03 (6)	70±5 (6)	26 (4)
0	Oxaloacetic, 5	5·19±0·72 (4)	1·26±0·33 (4)	82±6 (4)	10
0	Oxaloacetic, 25	5·95±0·56 (10)	1·26±0·33 (4)	121±5 (10)	14
+	Oxaloacetic, 5	4·51	1·71	90, 80*	—
+	Oxaloacetic, 20	4·44 (3)	1·94 (3)	120, 80	—
0	Pyruvic, 0·5	5·34 (3)	0·97 (3)	52 (3)	10
0	Pyruvic, 5	4·0 (2)	1·39 (2)	51 (2)	—
0	Pyruvic, 25	3·92±0·10 (4)	1·27±0·19 (4)	55±8 (4)	—
0	Malic, 20	6·5±0·30 (4)	1·12±0·25 (4)	51±6 (4)	18±7·5 (4)
0	Malic, 20; pyruvic, 0·5	5·24±0·50 (4)	1·34±0·22 (4)	74±6 (4)	20±5 (4)
0	Lactic, 20	4·25±0·44 (5)	1·64±0·28 (5)	62±3 (5)	—
+	Lactic, 10	3·22 (3)	1·88 (3)	58 (3)	—
0	L-Glutamic, 5	5·64±0·43 (4)	0·23±0·02 (4)	64±1 (4)	—
0	L-Glutamic, 20	5·35±0·25 (4)	0·4±0·26 (4)	70±7 (6)	—
0	D-Glutamic, 5	6·54±0·55 (4)	0·23±0·06 (4)	24±6 (4)	—
0	D-Glutamic, 20	5·53±0·39 (4)	0·20±0·05 (4)	22±7 (4)	—
0	Aspartic, 5	7·73 (3)	0·32 (3)	27±6 (10)	—
0	Aspartic, 20	7·65±0·46 (4)	0·40±0·09 (4)	29±3 (4)	5·5 (1)
0	Asparagine, 5	7·28±0·69 (4)	0·36±0·05 (4)	36±7 (6)	2·5 (1)
0	Asparagine, 20	6·56 (3)	0·68 (3)	33±4 (6)	5·0 (2)
0	γ-Aminobutyric, 20	4·69 (2)	0·23 (2)	36±4 (6)	6·0 (2)
+	γ-Aminobutyric, 5	2·92 (2)	0·92 (2)	65 (2)	—
+	γ-Aminobutyric, 25	3·37±0·9 (5)	1·08±0·25 (5)	74±10 (9)	—
+	Histidine, 20	3·81±0·45 (6)	1·02±1·0 (6)	72±6 (9)	—

* Respiratory rate was unstable, commencing at the value quoted and falling during the experiment.

Table 4. *Amino acids and tissue creatine*

Slices of guinea-pig cerebral cortex were incubated as described in Table 2 and after 40 min. creatine was extracted and determined as described in the Experimental section. Results on each line give mean values from three or four slices from the same animal, under each of the conditions *A* and *B*.

Additions (mM)		Free creatine of tissue (μmoles/g. wet wt.)	
To vessels *A* and *B*	To vessels *B* only	Vessels *A*	Vessels *B*
0	Glutamate, 20	2·02	2·0
Creatine, 1	Glutamate, 20	2·4	2·8
Creatine, 1	Aspartate, 20	3·4	3·9
Creatine, 1	β-Alanine, 20	2·16	2·14

substance in the brain do not appear to be available and it was therefore first determined in the brain fixed *in situ* to provide an indication of normal values (Table 5). The pyrophosphate so determined was largely lost from the tissue when it was prepared at ordinary temperatures for metabolic studies, but reappeared after incubation in media with glucose as the only oxidizable substrate. Addition of glutamate then caused loss of the pyrophosphate. There is thus no indication of its accumulating as a product of reactions involving glutamate.

(iii) The increase caused in the inorganic phosphate of cerebral slices by addition of glutamate raised the question of whether a change was being caused in the distribution of inorganic phosphate between the slice and the ambient medium; the experiments of Table 6 were carried out to answer this. They indicated that the inorganic phosphate of the medium also, increased in the presence of glutamate. This occurred whether or not phosphate was initially present in the medium; the increase in the glycylglycine medium was markedly greater than in a sodium hydrogen carbonate medium. These together with earlier observations (e.g. Table 3) show that glutamic acid causes a net increase in the inorganic phosphate of the incubation mixture and is not, for example, leading only to assimilation of inorganic phosphate from the medium to the tissue.

Table 5. *Inorganic pyrophosphate of cerebral tissues*

For methods of preparation and determination, see Experimental section. Values are either from duplicate determinations on single specimens, or when followed by S.D. are from the number of specimens given in parenthesis.

Preparation	Inorganic pyrophosphate (μmole/g. of tissue) Method (i)	Method (ii)
(*a*) Whole brain fixed *in situ* with liquid N_2	(Not applicable)	0·38±0·07 (4)
(*b*) Whole brain removed at room temperature	<0·01	0·004 (4)
(*c*) Cerebral cortex incubated for 40 min. in glucose–glycylglycine media	0·31, 0·35	0·27±0·03 (4)
(*d*) As (*c*), with glucose and 20 mM-glutamate	0, 0·10	0·02±0·02 (4)

Table 6. *Glutamate and the inorganic phosphate of the incubation medium*

Slices of guinea-pig cerebral cortex (80–100 mg.) were cut without contact with media and were placed in 3·5 ml. of the fluids named, which were either the ordinary oxygenated glucose media described in the Experimental section or differed from them only by the omission of KH_2PO_4 or addition of glutamate. After incubation for 40 min. tissues were removed and the media sampled for inorganic phosphate as described in the Experimental section; values quoted are the means of triplicate results, agreeing closely.

Buffer and initial inorganic phosphate (mM)	Inorganic phosphate gained by medium (μmoles/g. of tissue) Medium without addition	Medium with 20 mM-L-glutamate
Sodium hydrogen carbonate, 0	9·68	10·73
Sodium hydrogen carbonate, 1·3	12·0	13·1
Glycylglycine, 0	8·53	11·27
Glycylglycine, 1·3	12·1	17·1

(iv) Previous study showed phosphocreatine to be relatively stable in the presence of ground cerebral tissue, considerable loss occurring only on adding adenosine diphosphate or a substance which could yield it, when the creatine-phosphokinase reaction occurred (Narayanaswami, 1952; Ennor & Rosenberg, 1954). However, a reaction involving glutamate had not specifically been sought, and this possibility was therefore examined. With ground guinea-pig cerebral cortex at 5–20 mg./ml. in glycylglycine-buffered solutions at pH 7·4 with mM-$MgSO_4$ and 2·5 mM-phosphocreatine, glutamate at 20 mM caused no change in phosphocreatine loss. This remained true when the following additions (mM) were made: adenosine monophosphate, 0·3; inosine, guanosine and cytosine monophosphates, 0·5 and 5; NH_4Cl, 20; KCl, 100. Phosphocreatine synthesis was examined in mixtures containing (mM): glycine buffer, pH 8·9, 100; $MgSO_4$, 20; creatine, 7. With tissue at 20–40 mg./ml., 20 mM-glutamate did not affect the phosphocreatine synthesized or the inorganic phosphate released; synthesis proceeded at about 270 μmoles/g./hr. with tissue at 20 mg./ml.

Ammonia, glutamate and the speed of change in tissue phosphocreatine

The findings described emphasize glutamine synthesis as being the major known reaction which consumes energy-rich phosphate and in which glutamate participates. To appraise the adequacy of this reaction as a basis for the observed changes in phosphocreatine, an important characteristic is the speed at which loss of phosphocreatine occurs on addition of glutamate. This has been determined by incubating slices of guinea-pig cerebral cortex in media which initially contained only glucose as substrate. After phosphocreatine had reached a stable value, the media then received glutamate, ammonia or both, and tissues were removed for analysis at brief intervals after the additions. Fig. 1 shows the resulting changes in phosphocreatine and inorganic phosphate to be largely complete in 2 min. By fixing tissues at intervals of 30 sec., addition of either glutamate or ammonia was found to cause loss of about 25% of the tissue's phosphocreatine in 1 min. With both substrates, over half the phosphocreatine was lost in this time, corresponding to a change of 56 μmoles/g. of tissue/hr. Although ammonia accelerated the loss of phosphocreatine caused by glutamate, the final extent of the loss, after 10 min., was not appreciably altered. This is consistent with previous findings (Krebs, 1935; Weil-Malherbe, 1936) that glutamate can itself provide ammonia for glutamine synthesis.

Cocaine affects ion movements in neural tissues (Shanes, 1958) and at 20 μM inhibits the metabolic response of cerebral tissues to electrical pulses (Bollard & McIlwain, 1959). Because of the suggestions mentioned at the beginning of this paper that glutamic acid might affect tissue phosphates secondarily to an action on ion movement, experi-

ments analogous to those of Fig. 1 were carried out in the presence of cocaine. Phosphocreatine breakdown was not affected by 20 μM-cocaine; 75 μM-cocaine caused a 30% depression. These experiments therefore supported the view that glutamate–phosphate interactions were at a metabolic level.

Rate of glutamine formation. Previous studies of glutamine synthesis by cerebral tissues (Krebs, 1935; Weil-Malherbe, 1936) employed experimental periods of 20 min. to 4 hr. and concerned the total glutamine content of the tissue plus incubation fluid. The synthesis has since been examined in briefer periods with ground tissues or enzyme systems (Elliott, 1951; Krishnaswamy, Pamiljans & Meister, 1960), but data on glutamine production in intact tissues while the phosphate changes of Fig. 1 are taking place do not appear to be available. Such determinations have therefore been carried out and results are shown in Fig. 2. In these experiments tissues were preincubated for 30 min. to allow resynthesis of phosphocreatine. Addition of glutamate was then followed by production of glutamine. Negligible synthesis occurred in the first minute; during the second, 0·9 μmole of glutamine formed/g. of tissue, corresponding to synthesis at 54 μmoles/g./hr. The rate in the next 2 min. corresponded to 18 μmoles/g./hr. Thus the time-course and maximum observed rate of glutamine formation closely paralleled those of the loss of phosphocreatine (Fig. 1), except for the initial lag in glutamine production. Fall in tissue ammonia was smaller than the concomitant gain in glutamine, which is consistent with the formation of ammonia from glutamic acid, referred to above.

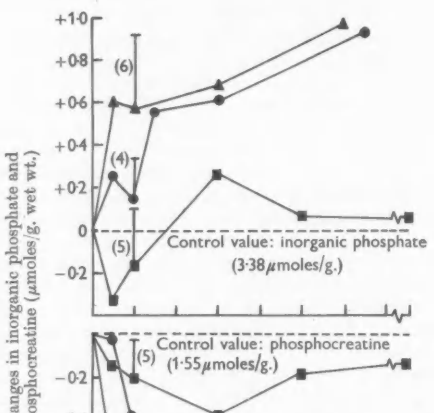

Fig. 1. Rapid changes in phosphates of guinea-pig cerebral cortex on addition of glutamates and ammonium salts. Tissues were incubated for 30 min. in glycylglycine media at 37°, and some removed for analysis at this time (zero time on the Figure). To others were added sodium glutamate, to make the solution 20 mM (●), ammonium chloride to 5 mM (■), or both (▲). After addition, at the intervals indicated, tissues were removed for analysis. Points give the mean of two or three values except when indicated by a number near them, when a line extends from the point, giving the S.D.

DISCUSSION

Phosphocreatine and glutamine synthesis

The relationship between Figs. 1 and 2 gives good evidence for the action of glutamic acid on tissue phosphates being due to the synthesis of glutamine (Elliott, 1951) according to (I):

(I) Glutamic acid + ammonia + ATP → glutamine + ADP + P

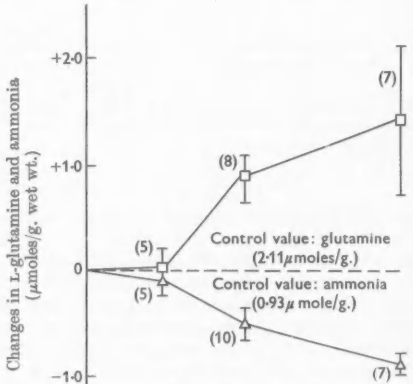

Fig. 2. Course of change in ammonia and glutamine on addition of glutamate to cerebral cortex. Slices of guinea-pig cortex of 90–110 mg. were incubated as described in Fig. 1, in vessels which contained 0·2 ml. of 0·5 M-sodium L-glutamate in side arms. After 30 min., the glutamate was tipped in; slices were removed at the intervals indicated and ground in trichloroacetic acid (see Experimental section) for analysis.

which, in association with creatine phosphokinase (of cerebral tissues: Narayanaswami, 1952), accounts for the change observed (II; Cr: creatine):

(II) Glutamic acid + ammonia + CrP → glutamine + Cr + P

A previous measurement (Achs, Balasz & Straub, 1952) of formation of glutamine and loss of adenosine triphosphate in cerebral tissues allowed the reaction to proceed for 40 min., or 10 times the period now found sufficient, and did not measure phosphocreatine; stoicheiometric relationship between glutamine and phosphate reacting were not found.

The glutamine-synthesizing enzyme of sheep brain has recently been purified and its action involves as intermediate an enzyme-bound, γ-carboxyl-activated glutamic acid (Krishnaswamy *et al.* 1960). Intermediates, formed from enzyme, glutamic acid, adenosine triphosphate and magnesium salts, were yielded by both D- and L-glutamic acid but that from the L-acid reacted more effectively with ammonia. In the present experiments D-glutamic acid also depleted tissue phosphocreatine, but did so rather less effectively than the L-acid (Table 2). Present observations on L-aspartic acid, which also depletes phosphocreatine, may receive explanation in terms of the very potent glutamic–aspartic transaminase of cerebral tissues, which can proceed at up to 2000 μmoles/g./hr. (Cohen & Hekhius, 1941; McArdle, Thompson & Webster, 1960), and thus provide glutamate from endogenous α-oxoglutarate and the aspartate supplied. By contrast, transamination between α-oxoglutarate and alanine or γ-aminobutyrate, though occurring in cerebral tissues, are markedly slower (of γ-aminobutyrate, about 10 μmoles/g./hr.: Baxter & Roberts, 1958) and the depletion now observed in phosphocreatine is much less (Table 3). β-Alanine has also been noted as possibly reacting with α-oxoglutarate, at a still lower rate; its effect in lowering phosphocreatine in the present experiments was the smallest of the compounds which so acted.

If the increase in phosphocreatine observed to follow incubation of cerebral tissues with phenylalanine is related to the findings of the preceding paragraph, inhibition of the glutamine-synthesizing system is suggested, but has not been examined.

Respiration with amino and keto acids

The actions of related keto and amino acids on tissue respiration and phosphates can be compared. The diminution in phosphocreatine which occurred when L- or D-glutamic acid, or aspartic acid or alanine was added to glucose-containing media did not take place with addition of the corresponding keto acids: α-oxoglutaric, oxaloacetic or pyruvic. On the other hand, α-oxoglutaric acid and oxaloacetic acid increased respiration of tissues in glucose media, as did L-glutamic acid and aspartic acid; D-glutamic acid and alanine did not. This suggests that the ability of the amino acids to act as oxidizable substrates depends on their conversion into the keto acids and is independent of their effects on phosphocreatine.

L-Glutamic acid is the only amino acid appreciably increasing respiration in the absence of glucose (Weil-Malherbe, 1936; Table 3), and the formation of glutamine may play an essential part in this. Reaction (III) can be regarded as including reaction (I), glutamic dehydrogenase and oxidative phosphorylation:

(III) 2 Glutamic acid + [O] → α-oxoglutarate + glutamine

It provides a source of oxoglutarate independently of glucose or its intermediates.

D-Glutamic acid, as noted, is less capable of participating in reaction (I) and may for this reason or through stereospecificity in glutamic dehydrogenase support a lesser respiratory rate. If aspartic acid, used as substrate in the presence of glucose, undergoes transamination with α-oxoglutarate derived from glucose, this may result in increased respiratory rate because oxaloacetate at lower concentrations (5 mM in Table 3) affords higher respiratory rates than does an equimolar concentration of α-oxoglutarate. The higher respiratory rate with oxaloacetate occurs in the presence or absence of glucose; the potency of the glutamic–aspartic transaminase makes understandable the formation (and preferential oxidation) of oxaloacetate. By contrast, transamination with alanine is less active, and pyruvate as only substrate does not afford respiratory rates as high as oxaloacetate: presumably for these reasons alanine does not have the effect on respiration which is given by aspartic acid.

Relation to ion movements

The additional potassium observed by Terner *et al.* (1950) to become associated with cerebral tissues on addition of glutamate to media in which they were respiring with glucose as substrate may receive explanation in terms of the foregoing results. Considering reaction (III) in relation to the ions involved, and supposing glutamic acid to enter the cell with K^+ ion 'as its cationic equivalent' (Terner *et al.* 1950), it is there converted into α-oxoglutarate, which does not greatly increase in amount in the tissue or medium and hence is assumed to be completely oxidized, and glutamine, which does increase in tissue and medium but which is not acidic. The cation could thus accumu-

late and possibly its uptake leads secondarily to the observed uptake of fluid, for the result of the ingress of both is to leave potassium salts at nearly their normal intracellular concentration (Pappius & Elliott, 1956).

This increase of tissue potassium is not, however, envisaged as making a major contribution, as such, to transport in the present tissue. This judgement is based on (1) the abnormalities which accompany the uptake of potassium promoted by glutamate: (*a*) the fluid uptake, (*b*) the fall in phosphocreatine and (*c*) the fall in membrane potential. Also (2) a mechanism based on reaction (III) is not sufficiently rapid. Cummins & McIlwain (1961) observed cerebral tissues to be capable of reassimilating potassium salts at the rate of 600 μequiv./g. of tissue/hr. The greatest increase in rate of K^+ ion assimilation observed (Pappius & Elliott, 1956) to be brought about by glutamate approximated to 15–20 μequiv./g./hr., a rate understandable if the assimilation is by reaction (III) and this is limited by reaction (I) to about 50 μmoles/g./hr. (see above). Further (3) the mechanism based on (III) requires uptake of glutamate from the environment of the tissue and the extrusion of glutamine. This does not occur in the brain *in vivo* at appreciable rates; those observed by Adams, Harper, Gordan, Hutchin & Bentinck (1955) corresponded to 2 μmoles/g. of brain/hr. An aspect of these findings which does appear significant in relation to normal ion transport is that the cation which accumulates on addition of glutamic acid is the K^+ ion. This takes place in spite of the preponderance of Na^+ ions in the tissue environment, and thus requires a specific explanation.

SUMMARY

1. Slices of mammalian cerebral cortex resynthesized phosphocreatine on incubation in media containing (5–25 mM) glucose, or lactic acid, or pyruvic acid, or malic acid or oxaloacetic acid. A lesser resynthesis occurred with α-oxoglutarate, and little or none with glutamic acid, aspartic acid and γ-aminobutyric acid as the only oxidizable substrates.

2. The concentrations of phosphocreatine produced in the tissue in the presence of glucose were diminished by the further addition of 5–25 mM-D- or L-glutamic acid, L-aspartic acid or γ-aminobutyric acid, asparagine, alanine or β-alanine, but were slightly increased by the further addition of 25 mM-L-methionine or L-phenylalanine.

3. Respiratory rates were increased, in the presence of glucose, by α-oxoglutarate and oxaloacetate but were diminished by the further addition of L-phenylalanine or L-tryptophan.

4. The depression of phosphocreatine by L-glutamate was not prevented by the addition of creatine to the medium. Also creatine assimilation was not affected by L-glutamate, L-aspartate or β-alanine.

5. Tissue inorganic pyrophosphate, normally at 0·2 μmole/g., was diminished by addition of L-glutamate.

6. Creatine-phosphokinase activity in cerebral homogenates was unaffected by the addition of glutamate or several nucleotide phosphates, when observed with phosphocreatine and adenosine diphosphate as initial reactants. Phosphocreatine synthesis by cerebral homogenates was also unaffected by the addition of L-glutamate.

7. The rate of phosphocreatine breakdown in cerebral slices on the addition of 20 mM-L-glutamate was about 20 μmoles/g./hr. and was not lowered by the addition of 20 μM-cocaine. Simultaneous addition of 5 mM-NH_4^+ ion and L-glutamate more than doubled this rate.

8. The rates of increase of tissue glutamine and loss of ammonia on addition of 20 mM-glutamate were similar to those for the loss of phosphocreatine and increase in inorganic phosphate, indicating that these changes were due to glutamine synthesis.

We are grateful to Mr A. McNeil for assistance during these investigations, which were carried out during the tenure by R. J. W. of a Medical Research Council Studentship.

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An Electrophoretic Analysis of Protein Extracts from Normal and Dystrophic Ovine Muscle

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Various extraction techniques have allowed the study of specific proteins found in muscle tissue, and the early work of Jacob (1947) demonstrated the usefulness of electrophoretic analysis in the study of these proteins. Such methods have been used both in fundamental studies of muscle proteins (see reviews by Perry, 1960; Szent-Gyorgyi, 1960) and in studies of pathological conditions of muscle (Azzone & Aloisi, 1958).

A degenerative myopathy affecting young lambs has been recognized for many years and referred to as nutritional muscular dystrophy, 'white muscle disease' or 'stiff lamb disease'. This disease is distinguished from infantile forms of muscular dystrophy in man by its non-progressive character, the capacity for complete regeneration of affected muscles, the absence of any evidence of familial predisposition and an apparent direct association of the ovine disease with maternal dietary factors. Defects in vitamin E metabolism have previously been recognized as important causative factors in the ovine disease (see review by Blaxter, 1955), but more recent evidence suggests that selenium metabolism may also be involved (Muth, Oldfield, Remmert & Schubert, 1958). There is also some evidence suggesting that a succinoxidase inhibitor in certain feeds may be of importance in the pathogenesis of this disease (Cartan & Swingle, 1959).

In order to characterize the changes occurring in the protein constituents of muscles in this disease, an electrophoretic analysis of extracts from affected and normal muscles has been made. This report describes specific changes in the electrophoresis patterns of these protein extracts that are shown to occur consistently in this disease.

MATERIALS AND METHODS

A high incidence of muscular dystrophy can be produced in lambs by feeding certain types of hay to their dams during pregnancy and immediately after parturition (Young, Hawkins & Swingle, 1961). Tissues for the observations described in this report were obtained from lambs affected with this experimentally induced muscular dystrophy and from normal lambs whose dams had received a hay known not to produce the disease.

Muscular dystrophy was detected in affected lambs by elevations in the quantity of glutamic–oxaloacetic transaminase occurring in their serum. For this purpose, serum samples were drawn from all lambs at 7, 14 and 21 days of age, and at the time of slaughter, and subjected to the test procedure described by Cabaud, Leeper & Wroblewski (1956). The close correlation that exists between increases in serum glutamic–oxaloacetic transaminase and the early changes of muscular dystrophy in lambs has previously been reported (Blincoe & Dye, 1958; Swingle, Young & Dang, 1959). In this experiment all affected lambs had elevations of this enzyme above 2000 units between the age of

14 days and slaughter at 18–29 days. They also had clinical evidence of the disease and were found at slaughter to be grossly affected with muscular lesions. All normal lambs had activities of less than 130 units of the enzyme on all sampling occasions, had no clinical evidence of the disease and were not grossly affected.

Affected and normal lambs were killed by bleeding at ages from 18 to 29 days. Specimens of skeletal muscle were obtained from each of two areas in each lamb: namely, a portion of longissimus dorsi muscle in the lumbar region, and the main belly of the quadriceps femoris muscle of one hind limb. The muscle specimens, each weighing 100–150 g., were first trimmed of excess of fat and connective tissue, briefly washed in water, placed in 100 ml. of water in a plastic bag and frozen in a solid carbon dioxide–ethanol bath. When ready for use, the tissues were allowed to thaw and sufficient phosphate buffer was added to give 100 ml. of buffer (0·4 M, pH 6·7)/75 g. of muscle. Muscles were then homogenized by grinding in a blender. An ice bath was used to ensure that the muscle temperature during blending did not exceed 3°. The tissues were homogenized for a period of 20 sec. and allowed to cool for 40 sec., and the cycle was then repeated until a total homogenization time of 5 min. had been completed. Muscle homogenates were then held at 3° for 24 hr., after which an extract was expressed through cheesecloth. Expressed muscle extracts were dialysed against phosphate buffer (Miller & Golder, 1950), I 0·2 at pH 7·0. After dialysis the extract was centrifuged at 35 000g for 10 min. and the supernatant fluid, then having a conductivity approximately that of the buffer used in dialysis, was employed in electrophoresis and ultracentrifuging.

To assist in the identification of the components seen in electrophoresis patterns, additional material from one extract was subjected to treatment with ATP before electrophoresis. ATP was added (10 mg./ml. of the extract) and the material incubated for 30 min. at room temperature. It was then centrifuged at 35 000g for 30 min. and the supernatant fluid subjected to electrophoresis.

Blood samples were obtained from lambs at the time of death, and the serum was removed after clotting had occurred at 4°. The sera were also dialysed against phosphate buffer (Miller & Golder, 1950), I 0·2 at pH 7·0, and then subjected to electrophoresis.

Electrophoresis was performed with a Perkin–Elmer model 38A at 1–2°. All curves shown were reproduced by photographic means. Mobilities were calculated by a standard equation (Alberty, 1948) and relative concentrations determined by planimetry.

Ultracentrifugal analysis was performed by the sucrose gradient method (Hogeboom & Kuff, 1954) in an attempt to separate protein components of the muscle extracts for further study. Gradients were prepared and placed in the centrifuge tube with a gradient-making device to produce linear gradients. The material was centrifuged in a Spinco model L centrifuge at 39 460 rev./min. in the swinging-bucket rotor SW 39 L. At the termination of centrifuging, samples were removed from the bottom of the centrifuge tube (Szybalski, 1960), 27–30 samples being taken from each tube. Approximate sedimentation constants were calculated by an equation of the form described by Hogeboom & Kuff (1954) with corrections made for 20° and water as described by Svedberg & Pedersen (1940).

RESULTS

The electrophoresis patterns of proteins from extracts of longissimus dorsi muscles from five normal animals are shown in Fig. 1. The patterns are very consistent and a quantitative measure of this consistency is given by the relative concentration of individual components in these extracts (Table 1). A similar consistency is evident in the electrophoresis patterns of proteins of extracts from the longissimus dorsi muscles of five dystrophic animals (Fig. 2), and this consistency is again

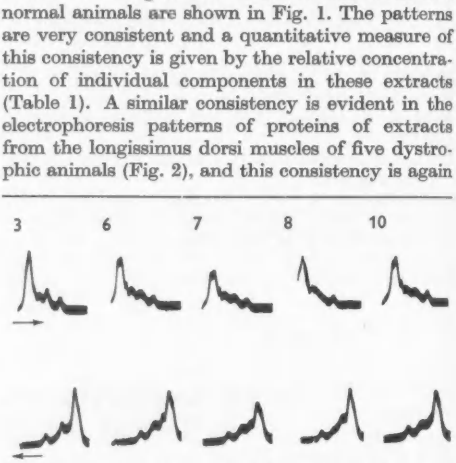

Fig. 1. Ascending (top row) and descending (bottom row) electrophoresis patterns of the proteins of longissimus dorsi-muscle extracts from five normal animals (runs 3, 6, 7, 8 and 10).

Table 1. *Relative concentrations of gradients from longissimus dorsi extracts*

Concentrations are expressed as a percentage of the total extract as determined by planimetry, with S.D. calculated on five samples in each case.

Component	Muscle-extract concentrations: Affected	Normal
1	6·4±1·0	2·0±0·7
2	23·3±3·5	9·3±2·0
3, 3a	30·8±4·2	20·8±1·9
4, 4a, 5, 6	38·0±3·8	67·4±1·9
Total	100	100

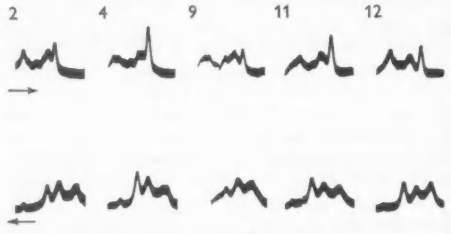

Fig. 2. Ascending (top row) and descending (bottom row) electrophoresis patterns of the longissimus dorsi-muscle extracts from five affected animals (runs 2, 4, 9, 11 and 12).

supported by the relative concentrations of individual components in these extracts (Table 1). The system of labelling components is shown in Fig. 3, and their mobilities are listed for reference in Table 2.

It is evident that there are differences between the normal and the dystrophic longissimus dorsi muscles. In the affected muscles, there is an increase in relative concentration of components 2 and 3–3a and a decrease in components 4–6. The mobilities of the components are relatively unaltered.

The electrophoresis results of the ATP test to assist in identification of extract components are shown in Fig. 4. Calculation of mobilities showed that centrifuging after the addition of ATP removed components 2 and 4a.

Extracts from quadriceps femoris muscle were also examined electrophoretically. Results similar to those shown for longissimus dorsi-muscle extracts were obtained. There is an increase in concentration of components 2 and 3–3a and a decrease in components 4–6 in extracts from affected muscles as compared with normal ones. An example of these results can be seen in Fig. 5.

To test the possibility that the decrease in concentration of components 4–6 in extracts from affected muscles might result in an increase in concentration of protein in blood serum due to the

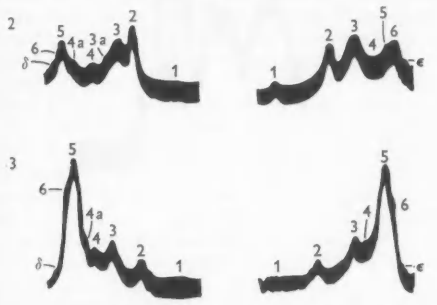

Fig. 3. System of labelling muscle extracts (top: affected, run 2; bottom: normal, run 3); (ascending and descending limbs).

Table 2. *Mobility values of gradients from longissimus dorsi extracts*

Mobility is expressed as 10^{-5} cm.^{2}/v/sec. S.D. was calculated on five samples in each case.

Component	Mobility: Descending limb	Mobility: Ascending limb
Affected muscles		
1	−6·37±0·32	−6·82±0·11
2	−3·82±0·29	−4·60±0·20
3	−2·31±0·18	−3·46±0·08
3a	—	−3·07±0·13
4	−1·32±0·15	−2·20±0·14
4a	—	−1·34±0·19
5	−0·64±0·16	−0·65±0·13
6	−0·28±0·15	−0·34±0·13
Normal muscles		
1	−6·62±0·10	−6·57±0·31
2	−4·15±0·35	−4·58±0·31
3	−2·31±0·21	−3·28±0·33
3a	—	−3·06±0·23
4	−1·32±0·14	−2·01±0·06
4a	—	−1·27±0·15
5	−0·60±0·23	−0·64±0·09
6	−0·23±0·08	−0·32±0·05

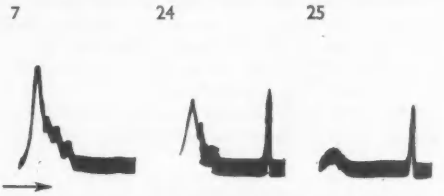

Fig. 4. A comparison of electrophoresis patterns of an extract from the longissimus dorsi muscle of a normal animal (7), the same extract after addition of 10 mg. of ATP/ml. of extract, incubation for 30 min. at room temperature, and centrifuging at 35 000*g* for 30 min. (24), and the pattern of 1% ATP alone (25).

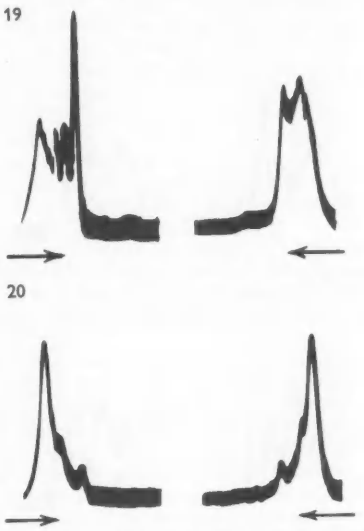

Fig. 5. Ascending and descending electrophoresis patterns of the proteins of quadriceps femoris-muscle extracts from an affected animal (19) and a normal animal (20).

release of these proteins from muscle cells, electrophoresis patterns of sera were obtained from dystrophic and normal animals (Fig. 6). The system of labelling is shown in Fig. 7. There is an increase in component 2 and a decrease in components 3 and 4 in the sera from dystrophic animals. The mobilities of serum components are recorded in Table 3.

In the ultracentrifuging studies, linear sucrose gradients were used which ranged from a 4–9 % to a 4–25 % with various lengths of time of centrifuging. With the latter gradient, and centrifuging for 150 min., some of the protein (possibly actomyosin, whose $S_{20,w}$ is reported to be from 50 to a few hundred) was found to sediment completely. A well-defined fraction was evident near the bottom of the tube ($S_{20,w}$ about 21). The remainder of the protein fractions were grouped near the top of the tube ($S_{20,w}$ from 8 down to 1). With a 4–9 % gradient and longer periods of centrifuging of up to 8 hr. it has been possible to achieve some separation of components in the latter group.

DISCUSSION

The electrophoretic gradients of proteins from normal muscle extracts seen in this study bear close resemblance to those of normal resting muscle shown by Dubuisson (1950) and by Crepax (1951).

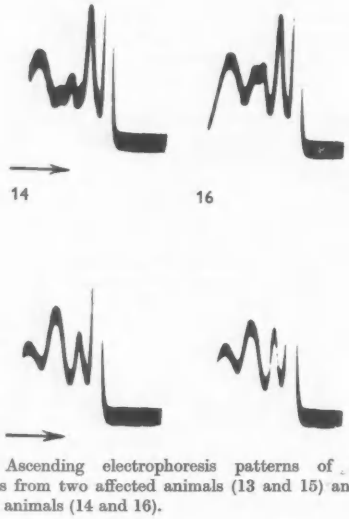

Fig. 6. Ascending electrophoresis patterns of serum proteins from two affected animals (13 and 15) and two normal animals (14 and 16).

These data from the literature suggest that one might tentatively identify the muscle-protein components (Fig. 3) in this report as follows: 1, myoalbumin; 2, actomyosin; 3, myosin; 3a, possibly contractin; 4 and 4a, unknown; 5 and 6, myogen. The experiment shown in Fig. 4 lends support to the assumption that component 2 is

Fig. 7. System of labelling serum proteins (top: affected, run 15; bottom: normal, run 16); (ascending limbs).

Table 3. *Mobility values of gradients from serum*

Mobility is expressed as 10^{-5} cm.^{2}/v/sec. (averages of two sera).

Component	Mobility of ascending limb
Affected	
Albumin (1)	−3·92
α-Globulin (2)	−2·86
β-Globulin (3)	−1·79
γ-Globulin (4)	−1·16
Normal	
Albumin (1)	−4·06
α-Globulin (2)	−2·88
β-Globulin (3)	−1·81
γ-Globulin (4)	−1·30

actomyosin, since upon addition of ATP and centrifuging this component was lost [by superprecipitation or splitting to yield actin and myosin with the subsequent formation of highly polymeric precipitable F-actin (Perry, 1956)]. The serum components are: 1, albumin; 2, α-globulin; 3, β-globulin; 4, γ-globulin.

The ascending mobilities of Tables 2 and 3 show that serum albumin is not the same protein as component 1 of the muscle extract (myoalbumin). Apart from α-globulin of serum and component 3a of the muscle extract, and γ-globulin of serum and 4a of the extract, no components of serum and extract have mobilities even remotely similar to one another. In short, the rather large decrease in extract components 5 and 6 (myogen) does not result in an increase of any serum component, since there is no serum component with similar mobility. This is interesting since it is known that some enzymes which are apparently lost from muscle to the serum in dystrophy do persist for some time in the serum undegraded (Blincoe & Dye, 1958; Blincoe & Marble, 1960; White & Hess, 1957; Zierler, 1958; Dreyfus, Schapira & Schapira, 1958). These enzymes probably represent only a small part of the loss of the myogen fraction. The relatively large amount of protein lost from this fraction is not accounted for. The results seem to suggest that the turnover or degradation of the bulk of the myogen protein lost from muscle tissue may be rather rapid.

In some pathologically similar myopathies occurring naturally in man, in a certain strain of mice and in vitamin-E-deficient laboratory animals, electrophoretic analysis has revealed alterations in the concentrations of various proteins from affected muscle. A comparison of those published results with the data reported in this manuscript reveal certain differences. Azzone & Aloisi (1958), Aloisi (1951) and Aloisi, Ascenzi & Bonetti (1953) have reported that in avitaminosis E of rabbits the myosin content of muscle-fibre extracts decreased, and in advanced dystrophy there was an increase in F-actomyosin in their fraction I extracts. Corsi, Gallucci & Bargellini (1959) report that extracts of isolated myofibrils of vitamin-E-deficient rabbits showed by electrophoresis an increase of actomyosin over extracts from normal animals. Horvath (1958) reported that in both human dystrophy as well as in an inherited form of muscular dystrophy in mice there is a decrease in the myosin fraction of muscle extracts. A decrease in myosin in hereditary dystrophy of mice was also reported by Oppenheimer, Terry, Forsyth & Milhorat (1959). These workers found an increase in myoalbumin as well. Extracts of dystrophic muscle have been reported by Sarteschi & Fabiani (1958) to show an increase in the myoalbumin fraction, and in extracts from biopsies of human dystrophy Corsi & Buscaino (1958) have reported a decrease in myosin and a variable increase or decrease in actomyosin.

The data reported here differ in certain respects from the results reported by others for these various myopathies. In general the reports in the literature have indicated: (1) a decrease in myosin in extracts from dystrophic muscles as contrasted with the increase reported here, (2) an increase in myoalbumin which is similar to that reported here, (3) in some cases a decrease in myogen which is similar to that reported here, and (4) a variable change in actomyosin as contrasted with the consistent increase of that component revealed by the present data.

Extraction systems used by various investigators have differed, and valid, quantitative comparisons cannot be made. In our work, and in certain other cases, no attempt has been made to determine differences in the muscles but rather only to compare the relative concentrations existing in the extract at the time of electrophoresis. Comparison of the total protein concentration (260/280 method of Warburg & Christian, 1941) in the extracts of normal and dystrophic muscle reveals that no significant difference exists (normal, $12{\cdot}0 \pm 1{\cdot}3$ mg. of protein/ml.; dystrophic, $13{\cdot}0 \pm 1{\cdot}7$ mg. of protein/ml.). This indicates that the differences in relative concentration (Table 1) reflect absolute differences in concentration in the extracts. Whether the variations in concentration that have been observed between normal and dystrophic muscle extracts reflect actual concentration differences in the muscles themselves has not been determined and is therefore not reported. Since the ovine disease under consideration here apparently differs from the progressive human and hereditary mouse myopathies in many ways, it is not surprising that the changes in the proteins seen in extracts from dystrophic muscle are apparently different in these diseases. Direct comparisons should be made under similar extraction conditions to determine whether the apparent differences among protein extracts in these diseases are real.

Since this disease is characterized by a capacity for complete regeneration of affected muscles, it would be profitable to examine electrophoretically, at various stages of recovery, extracts from muscles of lambs known to have been affected with the disease. Further, since the disease is known to be directly associated with maternal dietary factors, it seems desirable to compare the development of foetal muscle proteins from foetuses of ewes on a normal diet and those on a diet known to cause this disease.

SUMMARY

1. A comparison by electrophoretic analysis of extracts from normal and dystrophic ovine muscle has shown qualitative and quantitative differences in the protein composition between normal and affected muscle extracts.

2. There is an enhancement in two components which are thought to be actomyosin and myosin, and a marked decrease in a third component thought to be myogen in extracts from affected longissimus dorsi and quadriceps femoris muscles. These same extracts show an increase in a small, fast-moving component which is probably myoalbumin.

3. Electrophoresis differences were also observed in serum proteins from normal and affected animals. A marked increase in α-globulin and a decrease in the β-globulin and γ-globulin fractions were seen.

4. No increase in any serum component could be attributed to the decrease in the muscle-myogen fraction by the criterion of electrophoretic mobility. Similarly, muscle myoalbumin was not found to be the same protein as serum albumin by this criterion.

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Spectrophotometric Determination of β-Alanylhistidine Peptides in Ox-Muscle Extract

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Several workers have described chromatographic and microbiological methods for determining carnosine in muscle and in muscle extracts from various animals (Williams & Krehl, 1952; Wood & Bender, 1957; Davey, 1957; McManus, 1957, 1960). However, these methods either are too time-consuming and laborious for routine analyses or do not give reliable results. The present paper describes an accurate spectrophotometric method which is reasonably rapid and applicable to routine analyses.

EXPERIMENTAL

Meat extract. The commercial product known as 'no. 1 extract' or merely as 'meat extract' (Wood & Bender, 1957) was used.

Spectrophotometer. The spectrophotometric determinations were made with a Beckman model DU spectrophotometer in 1 cm. cells.

Chromatography. Paper chromatography was carried out by the ascending technique on Whatman no. 1 paper sheets (23 cm. × 25 cm.), with butan-2-one–propionic acid–water (75:25:30, by vol.) as solvent (Clayton & Strong, 1954).

The technique described by Hamilton & Anderson (1959) was employed for the column chromatography. The data for the column were: internal diameter, 0·9 cm.; length, 100 cm.; resin, Amberlite IR-120 II CG, fractionated according to the method of Moore, Spackman & Stein (1958) (fraction D consisting of particles $56 \pm 9\ \mu$ in diameter was used); flow rate of buffers, 18·8 ml./cm.²/hr.

Precipitation of peptides. A solution containing about 0·2 g. of carnosine or β-alanylhistidine peptides (3·30 g. of meat extract) in about 50 ml. of water was mixed with 5 ml. of 10% (w/v) *m*-digallic acid and 1–2 drops of silicone antifoaming agent. It was shaken for 10 min. in a mechanical shaker and then placed in a refrigerator for 2 hr. It was made up to 100 ml. with water and a portion centrifuged at 2500 rev./min. The supernatant liquid was filtered through Whatman no. 42 filter paper and 2 ml. of the solution transferred to a 50 ml. centrifuge bottle. With stirring by a magnetic stirrer were added in this order: 5 drops of N-HCl, 10 ml. of a saturated aq. $HgCl_2$ containing 47% (w/v) sodium acetate trihydrate and 6·7% (w/v) NaCl, and 5 ml. of aq. 4% (w/v) $Na_2B_4O_7,10H_2O$. It was set aside at room temperature until the precipitate had settled and then centrifuged at 2500 rev./min. for 30 min. All the supernatant liquid was sucked off through a thin capillary tube, and 5–6 drops of conc. HCl were allowed to fall on the precipitate. After stirring with a magnetic stirrer, about 5 ml. of water was added and the mixture saturated with H_2S. The precipitate was filtered off on Whatman no. 41 filter paper and the filtrate and washings were evaporated to dryness on a boiling-water bath. The residue was taken up in 6 ml. of 6N-HCl and after addition of a granule of Hengar silica (supplied by The Hengar Co., Philadelphia, Pa., U.S.A.) boiled for 8 hr. under reflux. The hydrolysate was dried on a boiling-water bath and allowed to cool, and the residue taken up with 10·0 ml. of water.

Separation of β-alanine from the α-amino acids. A mixture of 1 g. of basic Al_2O_3 (chromatographic grade) and 1 g. of freshly prepared $Cu(OH)_2$ was introduced into a glass adsorption column (28 cm. × 0·9 cm. diameter). The column was washed with 50 ml. of water by gentle suction. The hydrolysate (2 ml.) was then applied, followed by water so that the total volume of hydrolysate and water sucked through was just short of 50 ml. The volume was made up to 50 ml. The β-alanine in 25 ml. of this solution was oxidized with acidic $KMnO_4$, and the 2:4-dinitrophenylhydrazone prepared (Szalkowski, Mader & Frediani, 1951). The dried dinitrophenylhydrazone was dissolved in successive 5 ml. portions of methyl Cellosolve (ethylene glycol monomethyl ether) and made up to 50 ml.

Colour development and spectrophotometric measurement. A sample (10 ml.) of the solution was transferred to a 100 ml. graduated flask, 50 ml. of water at 20° added, and the flask placed in a water bath maintained at 20° for 10 min.; 5 ml. of 5N-NaOH was added and the mixture was diluted to volume with water at 20°. After thorough shaking the absorption was read in a Beckman DU spectrophotometer at 570 mμ in a 1 cm. cell. The instrument was adjusted to give zero absorption with a solution obtained by placing 10 ml. of methyl Cellosolve and 5 ml. of 5N-NaOH in a 100 ml. volumetric flask and making up to volume with water at 20°. With a factor obtained from a standard solution of β-alanine, the amount of β-alanine present in the hydrolysate was calculated. A blank prepared by carrying out all the operations described above on 25 ml. of water must be deducted both from the reading of the standard carried out to obtain the factor and from the reading of the sample under examination.

Table 1. *Analyses of meat extracts by the spectrophotometric and column-chromatographic methods*

Values are given as percentages of β-alanylhistidine peptides.

Sample	No. of analyses	Range	Average
Spectrophotometric method			
Argentine 1955	9	6·61–6·90	6·73
Argentine 1959	2	7·19–7·35	7·27
Paraguay 1959 (1)	14	5·50–6·18	5·83
Paraguay 1959 (2)	2	6·60–6·90	6·75
Paraguay 1959 (3)	2	6·50–6·60	6·55
Paraguay 1959 (4)	2	6·53–6·60	6·56
Tanganyika 1959 (1)	2	5·10–5·25	5·17
Tanganyika 1959 (2)	2	5·82–5·85	5·83
Tanganyika 1959 (3)	1	6·10	6·10
Kenya 1959 (1)	4	5·90–6·00	5·95
Kenya 1959 (2)	2	6·70–7·00	6·85
Column-chromatographic method			
Argentine 1955	4	6·50–6·90	6·65
Argentine 1959	2	7·22–7·30	7·26
Paraguay 1959 (1)	2	5·44–5·69	5·56

RESULTS

To determine the accuracy and the specificity of the proposed method, we added various amounts of pure carnosine (F. Hoffmann–La Roche and Co. Ltd., Basle, Switzerland) to a commercial meat extract whose content of β-alanylhistidine peptides had previously been determined by column chromatography. The recovery was almost quantitative. For instance, the recovery of 15·54 mg. of carnosine added to 0·5 g. of meat extract containing 6·8% of carnosine was 15·22, 15·44, 15·66 mg., representing a mean recovery of 99·35% and a standard error of ± 1·15; the recovery of 77·7 mg. of carnosine added to 2·5 g. of the same meat extract was 75·0, 77·2, 78·3, 78·3, 79·4 mg. (mean recovery of $99{\cdot}92 \pm 1{\cdot}90$%).

Analysis of 11 samples of meat extract from various sources yielded 5·6–6·6% (average 6·2%) of β-alanylhistidine peptides. Further, we found a

very good agreement between the results obtained by the proposed method and those obtained by the column chromatography (see Table 1) which showed that the peptides consisted of 90% of carnosine and 10% of anserine.

The precipitate obtained with the $HgCl_2$ reagent was dissolved in a few drops of 5% (w/v) NaCN solution and chromatographed on resin columns. Fig. 1 shows that the precipitate in question contains predominantly carnosine with anserine in much smaller amount, as well as neutral and acidic amino acids, which emerge first from the columns. To confirm the identity of the peaks, the precipitate was hydrolysed with 6N-HCl and the hydrolysate chromatographed on a resin column. Fig. 2 shows the β-alanine, histidine and 1-methylhistidine peaks.

Substances that, after oxidation with $KMnO_4$, can form insoluble dinitrophenylhydrazones with 2:4-dinitrophenylhydrazine interfere in the determination of β-alanine. Histidine and the neutral and acidic amino acids that were present in our hydrolysate did this. A weighed amount (100 μg.) of each amino acid tested or of a mixture of them gave values corresponding to 10 μg. of β-alanine. These interfering substances are, however, removed by passing the hydrolysate through the $Cu(OH)_2$–Al_2O_3 column. The presence of alumina not only facilitates the filtration, but also serves to retain any traces of other interfering substances, such as carbohydrates. Fig. 3 shows the disappearance of the interfering amino acids, after passage through the column; only trace amounts of neutral and acidic amino acids are still present (peak no. 1), and peaks nos. 2 and 3 have been identified, as creatinine and NH_3, respectively, which do not interfere.

The blue colour produced by the dinitrophenylhydrazone in alkaline medium quickly turned reddish on solution of the dinitrophenylhydrazone precipitate in acetone; it remained unchanged for at least 2 hr., even on exposure to light, when the precipitate was dissolved in methyl Cellosolve.

It is very important that the concentration of H_2SO_4 in the solution from which the dinitrophenylhydrazone is precipitated should be constant, since it markedly affects the precipitation. It is also advisable that, for each new batch of reagents, tests with known amounts of β-alanine should be carried out.

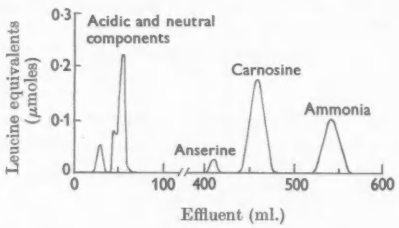

Fig. 1. Ninhydrin-positive components of the precipitate obtained from meat extract by means of the $HgCl_2$ reagent. The precipitate was dissolved in a few drops of 5% (w/v) NaCN solution and diluted to 10 ml. with 0·15M-citrate buffer, pH 5, and 2 ml. was put on the column. The eluting buffer for the first 180 ml. was sodium citrate (0·15M; pH 5) and sodium citrate (0·2M; pH 5) for the remainder.

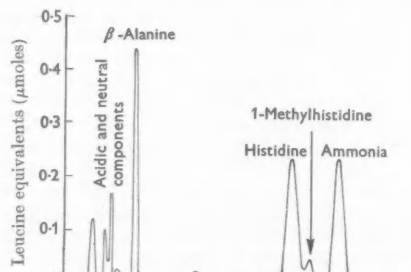

Fig. 2. Ninhydrin-positive components of the precipitate obtained from meat extract by means of the $HgCl_2$ reagent. The precipitate was dissolved in 6 ml. of 6N-HCl, hydrolysed for 8 hr., dried on a boiling-water bath and taken up in 10 ml. of 0·15M-citrate buffer, pH 5, and 2 ml. was put on the column. The eluting buffer for the first 180 ml. was sodium citrate (0·15M; pH 5) and sodium citrate (0·2M; pH 5) for the remainder.

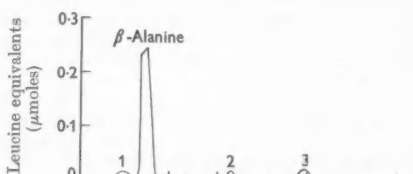

Fig. 3. Ninhydrin-positive components of the aq. eluate from the $Cu(OH)_2$–Al_2O_3 column. This was concentrated to 10 and 2 ml. put on the resin column. The eluting buffer for the first 180 ml. was sodium citate (0·15M; pH 5) and sodium citrate (0·2M; pH 5) for the remainder. Peak 1 corresponds to a trace amount of neutral and acidic amino acids, peaks 2 and 3 to creatinine and NH_3 respectively.

DISCUSSION

The method described is specific for β-alanylhistidine peptides, since, of the substances containing the histidine group that are precipitable by the mercuric chloride reagent, only those which yield β-alanine or subsequent hydrolysis are determined. In biological products this group includes only carnosine and anserine.

SUMMARY

1. A reasonably rapid and accurate method is described for the determination of β-alanylhistidine peptides in biological materials.

2. The peptides are precipitated with a mercuric chloride reagent, the precipitate is hydrolysed with 6N-hydrochloric acid, and the β-alanine formed is oxidized to a product which forms a dinitrophenylhydrazone.

3. The recovery of the carnosine added to a meat extract was 99 ± 1·90%.

4. Meat extract from various sources contains 5·6–6·6% (average 6·2%) of β-alanylhistidine peptides containing 90% of carnosine and 10% of anserine.

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Rutilantin: an Antibiotic Substance with Antiphage Activity

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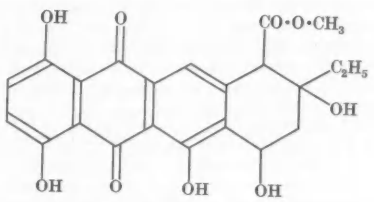
Asheshov, Strelitz, Hall & Flon (1954) examined 1000 actinomycete cultures for antiphage activity to determine whether bacterial viruses could be used as simulants of animal or plant viruses, since antiphage tests can be handled more easily and give more rapid results than antivirus tests. Of these cultures, 201 showed antiphage activity. One of them, which was bright red in colour (Serial No. A220), had a wide range of activity against phages of various strains of staphylococci, enterococci, *Bacillus cereus*, *Bacillus megatherium*, *Escherichia coli*, *Salmonella paratyphi* and *Vibrio cholerae*. Extracts prepared from cultures of this organism were also active against the viruses of Eastern and Western Equine encephalomyelitis, PR8 influenza, mumps and Y-SK poliomyelitis.

It was of interest to investigate the chemistry of the active substance, to which the name 'rutilantin' was given. It was recently shown by Ollis, Sutherland & Gordon (1959) that mild acid hydrolysis of rutilantin picrate, or of the purified base, yielded a bright-red crystalline aglycone ('rutilantinone'), and a water-soluble basic carbohydrate fraction, neither of which showed antibacterial or antiphage activity. Chemical investigations led to structure (I) for rutilantinone.



(I)

This constitution was of particular interest because it was later established by Brockmann *et al.* (1960) that it is identical with the structure of

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ϵ-pyrromycinone described by Brockmann & Lenk (1959*a*) derived from the cinerubins (Ettlinger *et al.* 1959) and also from pyrromycin (Brockmann & Lenk, 1959*b*), both of which are isolated from various streptomyces strains.

The present paper describes the isolation and partial purification of the active material.

EXPERIMENTAL

Culture conditions

Medium. This consisted of (g./l.) baker's yeast (20), glycerol (20), K_2HPO_4 (1·0), $MgSO_4,7H_2O$ (0·5). Trace elements were included, as follows (mg./l.): boric acid (57), $CuSO_4,5H_2O$ (157), $FeSO_4(NH_4)_2SO_4,24H_2O$ (1400), $MnSO_4,5H_2O$ (81), and $ZnSO_4,7H_2O$ (79). Silicone A (23 p.p.m.) and triamyl citrate (340 p.p.m.) were also present, as antifoam. Fermentations were carried out in 100 gal. stainless-steel fermentors at 24° for 96 hr. with a stirring rate of 250 rev./min. and an aeration rate of 16 ft.³/min.

Inoculum. This was produced in 10 l. aspirators containing 8 l. of yeast medium, stirred at 300 rev./min., aerated at 18 l./min., and incubated at 24° for 96 hr. The amount of inoculum added was 1% (v/v).

Assay

Diffusion assay. The agar diffusion technique, with *V. cholerae* and phage (Asheshov, Hall & Flon, 1955), was used. The medium used for the plates consisted of papain digest broth (Asheshov, 1941) containing agar (0·75%). The inoculum was a 4 hr. culture of *V. cholerae* in papain broth to which the phage (filtered free of bacteria) was added immediately before use. The plates were flooded with a mixture of host cells and phage in papain digest broth buffered to pH 7, the excess of fluid was pipetted off, and the plates were returned to the incubator for 30 min. Filter-paper disks (1·3 cm. diameter) were soaked in test solution, dried if an organic solvent was being used, and deposited on the plate. Plates were incubated for 18 hr. at 37°. Antiphage activity appeared as a zone of normal bacterial growth in the clear agar. Attempts to develop a reliable unit assay were unsuccessful because the zones obtained were irregular in shape, and had an uneven and not very well-defined edge; the slope of the plot of zone diameter against log concentration for this reason was not very reproducible. Results for diffusion assays are accordingly expressed as mean zone diameter (cm.) multiplied by 5·7. The figures thus obtained could only give a rough indication of the activity of a solution, and where a more quantitative assessment of antiphage activity was required, broth dilution tests were employed.

Broth dilution assay. The procedure was that of Asheshov, Hall & Flon (1955) with the demonstrable lysis of host cells in liquid medium as an indicator of normal phage activity, antiphage activity being indicated by growth of host cells. Tests were made in 5 ml. of broth of the following composition (/l.): papain digest (400 ml.), glucose (10 g.), NaCl (8·5 g.), bromocresol purple (10 mg.), trace elements (as already described); pH 7·3. *V. cholerae* cells (0·1 ml.) were suspended in broth, phage was added to each tube, and the tubes were incubated at 37° for 18 hr. The end point was the greatest dilution of active substance at which growth was normal, accompanied by liberation of acid indicated by change in colour.

Isolation

Preliminary experiments. At the end of the fermentation period, cultures showed antiphage zone diameters of 11·6–12·4 cm. No apparent loss of activity occurred on standing at room temperature at pH values between 2 and 10 for 12 hr. On centrifuging at neutral pH, a rather cloudy supernatant was obtained, but at pH 2 the supernatant was clear. The antiphage activity, as well as the red pigment, was present both in the mycelium and the supernatant. Tests showed that colour and activity could be transferred readily from the supernatant into various water-immiscible organic solvents, after making them slightly alkaline with dilute aqueous NH_3 (NaOH or KOH were not so convenient for this purpose, since they tended to form precipitates at pH values above 9, with irreversible loss of activity). Effective solvents included ethyl and *n*-butyl acetate, $CHCl_3$, trichloroethylene, and butan-1-ol. Light petroleum removed some of the colour, but none of the activity.

Active material could be extracted from mycelium with acetone or methanol. From this, it could be transferred to a water-immiscible solvent, such as $CHCl_3$ or trichloroethylene.

From trichloroethylene or $CHCl_3$, activity could be transferred back into aqueous solution by adding 1 vol. of light petroleum, extracting the mixture with 0·1 N-HCl, and neutralizing with dilute aqueous NH_3. This left only a trace of activity in the solvent mixture but a large amount of the colour remained, indicating that part of the red material is inactive.

Precipitation as picrate or reineckate. When an aqueous extract was treated with excess of 1% picric acid, or with ammonium reineckate, a bright-red precipitate was formed. The supernatant in either case was completely free of antiphage activity and showed only slight colour. This reaction accorded a convenient means of isolating the active material as solid.

Procedure for isolation as picrate

Culture (320 l.) was adjusted to pH 2 and centrifuged through a Sharples centrifuge.

Supernatant. The supernatant showed antiphage activity at 1:100 dilution. After adjusting to pH 9·5 with 2·5 N-NH_3 solution it was stirred vigorously for 5–10 min. with trichloroethylene (0·25 vol.). The emulsified mixture was passed at a rate of 150 l./hr. through an Alfa-Laval centrifuge, the bowl of which was assembled for emulsion separation. The aqueous phase, which was practically colourless and inactive, was rejected and the trichloroethylene phase evaporated down to 2–3 l. at 10 mm. in a van Heyningen (1949) distillation apparatus. The concentrate was active at a dilution of 1:5000, and the activity of the solid content 1:480 000. It was treated with 1 vol. of light petroleum (b.p. 80–100°), the mixture filtered if necessary, and extracted twice with 0·1 N-HCl (0·5 vol.). The combined HCl extracts were treated with excess of aqueous 1% picric acid, and the precipitate was filtered off and dried in air at 30°. Yield 40 g.

Mycelium. This was stirred for 20 min. with acetone (20 l.) and filtered by suction. The solid residue was stirred 20 min. with a further 10 l. of acetone, 2·5 N-NH_3 solution

being added to the mixture to bring the solution to a dark red. The mixture was filtered again and the solid residue rejected. The pooled acetone extracts were stirred with 1 vol. of trichloroethylene and 0·5 vol. water, sufficient aqueous NH_3 solution being added to bring the pH to 9·5, and the mixture was allowed to settle. The aqueous layer was siphoned off and re-extracted with 0·5 vol. of trichloroethylene. The spent aqueous phase was inactive, whereas the pooled organic extract was active at a dilution of 1:1500; activity of solids was 1:200 000. The volume of the solvent mixture was then reduced at 10 mm. to 6–8 l. The concentrate was treated with 1 vol. of light petroleum, and the mixture extracted with 0·5 vol. of 0·1 N-HCl. A red precipitate formed; this was filtered off, stirred with 0·1 N-HCl (1–2 l.) and treated with excess of aqueous 1% picric acid. The organic layer was then re-extracted with 0·1 N-HCl (0·5 vol.), and the pooled HCl extracts were treated with aqueous 1% picric acid. The picrate precipitates were filtered off and dried as previously. Yield 50 g.

Preparation of soluble salts from picrate

The antibiotic was readily converted into the water-soluble hydrochloride or citrate, as follows: Picrate (0·75 g.) was suspended in 15 ml. of $CHCl_3$, and a slow stream of NH_3 gas passed through the solution for 1–2 min. The mixture was centrifuged, and the supernatant washed three times with water (1 vol.), the first wash water being adjusted to pH 8. The $CHCl_3$ solution was treated drop by drop with a solution of HCl gas in ethyl acetate (2 N) until precipitation was complete, and the precipitate centrifuged off and washed with ether. The hydrochloride preparation thus obtained was light red in colour and showed antiphage activity at a dilution of $1:5 \times 10^6$ (i.e. 1 part in 5 million).

To prepare the citrate the hydrochloride was redissolved in water, adjusted to pH 6 with aqueous NH_3 solution and extracted twice with ethyl acetate (1 vol.). The extract was concentrated to about one-third vol. and treated drop by drop with saturated citric acid in dry ethyl acetate, until precipitation was complete. The bright-red precipitate was centrifuged down and washed with ether. Different batches of citrate preparations were active at dilutions varying between $1:5 \times 10^6$ and $1:7 \times 10^6$.

Yields of hydrochloride or citrate were approximately 20–30% of the picrate used.

PROPERTIES

Rutilantin is evidently a basic substance having little solubility in water at neutral pH. The citrate is soluble in water to the extent of approximately 1 mg./ml., and the hydrochloride much more soluble.

Stability. Aqueous solutions retain full activity for 12 hr. at room temperature at pH values between 1 and 10, but in N-HCl a slight fall in activity occurs. At 40° activity remains the same for 1 hr. at neutral pH, but is completely destroyed at pH 2 or 10. Above 40°, activity is rapidly lost, even at neutral pH.

Toxicity to mice. Citrate preparations were tested for toxicity by injecting them intraperitoneally into mice, 12 mice being used for each dose. From these experiments it was found that a citrate preparation of activity $1:5 \times 10^6$ had a LD_{50} of 45 mg./kg. Another preparation with antiphage activity $1:7 \times 10^6$ showed LD_{50} of 55 mg./kg.

Antibacterial spectrum. Tests of antibacterial activity were also carried out on solutions of the citrate by using the agar diffusion technique. At a concentration of 1:10 000 there was appreciable inhibitory action against the following organisms: *Aerobacter cloacae*, eight strains of *Micrococcus*, three strains of *Staphylococcus albus*, four strains of *Staphylococcus aureus*, *Sarcina lutea*, two strains of *Streptococcus pyogenes*, *Str. cremoris*, *Str. lactis*, *Str. faecalis*, two strains of *Str. agalactiae*, *Bacillus anthracis*, *B. megatherium*, *B. mycoides*, *B. cereus*, six strains of *B. subtilis* and *Salmonella typhi*. At a concentration of 1:100 000 the antibacterial activity was negative or doubtful. It would appear therefore that antiphage activity is at least 50 times as great as antibacterial activity.

DISCUSSION

Isolation of rutilantin in the solid form from large volumes of culture proved to be a relatively simple matter, advantage being taken of the partition between certain organic solvents and water at different pH values, and the insolubility of the picrate salt in water. The quantities of picrate obtained separately from the supernatant and the mycelium indicate that the active substance is distributed fairly evenly between the two phases of the culture.

Biologically, this substance is of fundamental interest on account of its high antiphage activity. In view of the earlier report of Asheshov *et al.* (1954) on antivirus activity, it would be of great interest to test the purified preparations against viruses. The mouse tests indicate it to be a toxic substance, but this may perhaps not necessarily exclude its ultimate therapeutic use, since the LD_{50} is still very high in relation to the antiphage activity.

SUMMARY

1. A simple method has been developed for the isolation and partial purification of rutilantin from cultures of an actinomycete (A 220).

2. The antiphage activity of the isolated material is at least 50 times its antibacterial activity.

3. The partly purified substance is toxic to mice.

We are indebted to Mr R. C. Codner for maintaining the culture used; to Mr G. A. Miller for carrying out the large-scale fermentations; to Mr R. W. Brimblecombe for his valuable help with the biological assays; and to Mr B. K. Kelly, the Director of the Antibiotics Research Station, for his encouragement of this work.

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The Oxidation of D-α-Hydroxy Acids in Animal Tissues

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It was realized by 1913 that the lactate formed by mammalian tissues from various hexoses is entirely of L-configuration (Levene & Meyer, 1913). Dakin & Dudley (1913*a*) and Neuberg (1913), however, showed that animal tissues form lactate from methylglyoxal, which, until the work of Lohmann and others in 1931–33 (e.g. Meyerhof, 1933), was thereupon believed to be an intermediate in glycolysis. That methylglyoxal gives rise to the 'unnatural' D-lactate caused surprisingly few misgivings. This reaction is now thought to be part of an amino ketone cycle (see Discussion).

It has been almost universal practice to use DL-lactate in experiments with animal tissues, the D-isomer being ignored on the assumption that it is metabolically inert, despite various reports to the contrary (e.g. Dakin & Dudley, 1913*b*; Abramson & Eggleton, 1927; Cori & Cori, 1929; Brin, Olson & Stare, 1952).

Huennekens, Mahler & Nordmann (1951*a*, *b*) reported that fresh, crude mitochondrial suspensions from rabbit liver and kidney could oxidize D-lactate, but that the L-enantiomorph was not oxidized unless the particles were supplemented with a 'factor', supposed to be a lactate racemase, from the soluble cytoplasm. Mahler, Tomisek & Huennekens (1953) continued this work, using both manometric and dye reduction (Thunberg tube) techniques; no evidence was obtained for the involvement of coenzymes in the formation of pyruvate from D-lactate, and attempts to obtain D-lactate oxidation in non-particulate systems were unsuccessful. Baker (1952) and Winitz *et al.* (1956) reported oxidation of D-α-hydroxy acids by rat-tissue preparations; again no coenzyme requirement was found. With kidney only the mitochondrial fraction was effective, and the slight activity in the soluble fraction of liver homogenates was perhaps due to glycollate oxidase (Kun, Dechary & Pitot, 1954), an enzyme virtually absent from kidney.

The present work has extended these various findings. Mitochondrial D-α-hydroxy acid dehydrogenase has been obtained in soluble form and partially purified. This communication is mainly devoted to the properties of this enzyme, which, it appears, should be added to the short list of mitochondrial 'cytochrome-reducing dehydrogenases' (Singer & Kearney, 1960).

Preliminary reports have appeared (Tubbs & Greville, 1959; Tubbs, 1960*a*), a detailed account has been given elsewhere (Tubbs, 1960*b*), and effects of metal-complex-forming agents on the dehydrogenase have been described (Tubbs, 1960*c*).

MATERIALS AND METHODS

Abbreviation. DEAE, Diethylaminoethylcellulose.

Water redistilled in Pyrex apparatus was used, and, when possible, AnalaR reagents were used. Solutions of substrates and labile compounds were stored at −10° unless prepared immediately before use. KCN solutions were neutralized with HCl.

Buffers. Tris buffers were prepared from the 'Sigma 121' product (Sigma Chemical Co., St Louis, Mo., U.S.A.) and HCl. Glycylglycine (Roche Products Ltd.) and KH_2PO_4 were brought to the required pH with KOH, as was $Na_4P_2O_7$ with HCl. Measurements of pH were made at room temperature with a Pye glass electrode and meter.

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Carboxylic acids. Calcium D- and L-lactate (California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A.) were passed through columns of Amberlite IR-120 or Zeo-Karb 225 (used in the hydrogen form after previous washing with NaOH), and the resulting acids neutralized with KOH. DL-α-Hydroxy-*n*-butyric acid (L. Light and Co. Ltd.) was converted with $BaCO_3$ into the barium salt, which was recrystallized twice from aqueous ethanol and then converted into the potassium salt by the procedure used for lactate. The D-isomers of α-hydroxy-*n*-pentanoic, α-hydroxy-*n*-hexanoic and α-hydroxy-γ-methylpentanoic acids, and both diastereoisomers of D-α-hydroxy-β-methyl-*n*-pentanoic acid, were obtained, as sodium salts, through the kindness of Dr C. G. Baker, National Cancer Institute, Bethesda, Md., U.S.A.; they had been prepared in the laboratory of the late Dr J. P. Greenstein (see Winitz *et al.* 1956). DL-α-Hydroxy-*n*-octanoic acid was prepared from DL-α-amino-*n*-octanoic acid (Kodak Ltd.) (Winitz *et al.* 1956); the sodium salt was recrystallized twice from acetone. Barium DL- and L-glycerate were kindly given by Professor F. Dickens, F.R.S.; the barium salts were converted into potassium salts by a slight excess of K_2SO_4 or by the procedure described for lactate. D-Malic and D-tartaric acid (California Corp. for Biochemical Research) and glycollic acid (Fluka A. G., Buchs, Switzerland) were neutralized with KOH. Tests with purified L-malate dehydrogenase, kindly carried out by Mr C. J. R. Thorne, showed that the malic acid was free from the L-enantiomorph. Barium D-3-phosphoglycerate (C. F. Boehringer und Soehne GmbH., Mannheim, Germany) was dissolved in dil. HCl and the potassium salt prepared as described for lactate. Lithium pyruvate was prepared from freshly distilled pyruvic acid [b.p. 71–73° (19 mm. Hg)] (Wendel, 1932). Sodium D-β-hydroxybutyrate had been made by Lehninger & Greville (1953).

Electron acceptors. 2:6-Dichlorophenol-indophenol (British Drug Houses Ltd.) was purified by the method of Savage (1957); the crystals were stored *in vacuo* in the dark, and stock solutions (0·57 mM in 10 mM-tris, pH 7·8) were kept at 4° for not more than 2 weeks. Methylene blue was a 'zinc-free' product (Merck, Germany). 5-Methylphenazinium methosulphate (phenazine methosulphate) was synthesized, with the kind help of Dr R. Hill, F.R.S., by the method of Singer & Kearney (1957) except that xylene was used as solvent; aqueous solutions (1%, w/v) were stored at −10° in darkness. Pyocyanine was prepared from phenazine methosulphate (McIlwain, 1937). Cytochrome *c* from horse heart (Keilin & Hartree, 1945) was further purified by the method of Margoliash (1954); solutions (0·5 mM) of the oxidized form were prepared by aeration at pH 2·5 and stored frozen at pH 7·8. FMN and FAD were obtained from Sigma Chemical Co., and NAD was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.

Sources of other materials. Sodium 5-ethyl-5-*iso*amylbarbiturate (amytal) (Eli Lilly and Co. Ltd.), Mepacrine hydrochloride (Imperial Chemical Industries Ltd.), ATP (Sigma Chemical Co.) and DEAE (0·59 m-equiv./g. dry wt.) (California Corp. for Biochemical Research). Hexokinase (stage V; Berger, Slein, Colowick & Cori, 1946) was kindly given by Miss R. M. Carpenter.

Protein determination. Protein concentration was determined in soluble preparations by the method of Warburg & Christian (1941). It was expressed as mg./ml. by multiplying $E_{280\,m\mu}$ by a factor (Layne, 1957) obtained from the ratio $E_{280\,m\mu}/E_{260\,m\mu}$. This ratio was never less than 0·85.

Mitochondrial respiration. This was measured in the Warburg apparatus, with air as the gas phase and KOH in the centre well.

Assay of D-α-hydroxy acid dehydrogenase. This was performed in a Beckman model DU spectrophotometer. Water from a thermostat was circulated through 'thermospacers' on either side of the cell compartment and kept the latter at 25°. In the standard assay the following were placed in a cell (light-path 1 cm.; final volume 3 ml.; final concentrations given): tris buffer, pH 8·5, 41·5 mM; D-lactate, 25 mM; 2:6-dichlorophenol-indophenol, 38 μM. Enzyme (0·01–0·3 ml.; 0·05–3 mg. of protein) was added and $E_{600\,m\mu}$ measured at 30 sec. intervals for 5 min. The mixture was brought to 25° before addition of enzyme and (when used) cyanide.

The unit is defined as that amount of enzyme which causes a decrease in $E_{600\,m\mu}$ of 0·01/min. after subtraction of the rate in the absence of added substrate; with dialysed and purified preparations this blank was negligible. Since the activity of freshly prepared extracts is greatly stimulated by low concentrations of cyanide (see below), the effect of including 0·5 mM-cyanide in the system should always be tested.

Preparation of mitochondria. Kidneys removed from rabbits immediately after death were put in ice-cold 0·25 M-sucrose. The preparation was carried out in a room at 4° and materials were kept close to 0°. After removal of capsule, medulla and fibrous tissue, the cortex was rinsed with 0·25 M-sucrose and finely cut with scissors. It was homogenized in 9 vol. of 0·25 M-sucrose by eight to ten strokes in a Potter–Elvehjem homogenizer (internal diameter of tube 25·0 mm.), the Perspex plunger (diameter 24·5 mm.) being rotated at low speed by a geared motor. Fractionation was carried out in 50-ml. tubes in swing-out buckets in a Major refrigerated centrifuge (Measuring and Scientific Equipment Co. Ltd.). The homogenate was centrifuged for 9 min. at about 350*g*. The mitochondria were separated from the supernatant by a total of approx. 41 000$g_{av.}$-min. (de Duve & Berthet, 1953), the centrifuge being set to run at 2600$g_{av.}$ for 15 min. They were then washed twice with 0·125 M-KCl, being sedimented at 41 000*g*-min. as before, and suspended in a volume of 0·25 M-sucrose equal to that of the cortex used. This procedure was also applied to kidneys of other species, but with liver the homogenizer pestle was not mechanically rotated and the mitochondria were washed with 0·25 M-sucrose.

When the mitochondria were required for the preparation of acetone-dried powder the second washing was omitted and the pellet was resuspended in a minimal quantity (about one-third of the cortex volume) of 0·25 M-sucrose.

Preparation of soluble D-α-hydroxy acid dehydrogenase. Acetone-dried powders were obtained by slowly adding mitochondrial suspensions to 15 vol. of continuously stirred acetone at −10°. After 2–3 min. the acetone was removed by centrifuging at −10° (250*g*; 3 min.) and decanting, and the powder washed twice on the centrifuge with acetone at −10°. It was dried in a desiccator (0·05 mm. Hg) containing P_2O_5, KOH and paraffin-wax shavings. The yield was about 2 g. from 100 g. of fresh kidney cortex and about 4·5 g. from 100 g. of liver. Stored in a desiccator at 4°, the powders gave fully active extracts even after many weeks.

The dried mitochondria were extracted with 20 mM-tris, pH 7·8 (1 ml./30–50 mg.), for 60–90 min. at 0°; the mixture was continuously stirred and care taken to break up lumps. The clear yellow supernatant after centrifuging at 8000*g* for 30 min. contained the enzyme.

Fractionation with $(NH_4)_2SO_4$ was carried out at 4°. Solid $(NH_4)_2SO_4$ was added to 32% saturation (Dixon, 1953). After stirring for 30 min. and centrifuging (3000*g*; 20 min.), the precipitate was discarded, and the supernatant brought to 42% saturation with $(NH_4)_2SO_4$ and stirred and centrifuged as before. The precipitate was dissolved in 20 mM-tris, pH 7·8. The resulting clear solution contained most of the dehydrogenase, but usually the material precipitated at 50% saturation with $(NH_4)_2SO_4$ was added and the combined solution dialysed overnight against the above tris buffer. Most experiments have been performed with enzyme purified to this stage.

Further purification has been achieved by chromatography on columns of DEAE at 4°. The DEAE was equilibrated with 20 mM-tris, pH 7·8, protein (125 mg./g. dry wt. of DEAE) dissolved in the same buffer passed into the column, and an increasing KCl gradient applied (Sober & Peterson, 1958). The enzyme emerged from the column when the KCl concentration in the eluate was 0·12–0·15 M. To concentrate fractions containing the enzyme, $(NH_4)_2SO_4$ was added to 60% saturation, the precipitate dissolved in a small quantity of the tris buffer, and the solution dialysed at 4° against the same buffer.

RESULTS

Oxidation of D-*lactate by particulate preparations*

Complete stereospecificity of liver and kidney particles for D-lactate, as reported by Huennekens *et al.* (1951*a*, *b*) and Mahler *et al.* (1953) was never observed; however, the D-enantiomorph was oxidized much more rapidly than the L. Typical experiments with rabbit-kidney and rat-liver particles are shown in Fig. 1. In other experiments citrate (0·33 mM) replaced fumarate as 'sparker'. It is not possible to compare the present results exactly with those of the authors mentioned above, since all oxygen uptakes reported by them were net values, the blank values, of unstated magnitude, having been subtracted.

In agreement with Huennekens *et al.* (1951*b*), pigeon-breast-muscle mitochondria, which were isolated and twice washed in 0·25 M-sucrose or 0·125 M-potassium chloride, both containing either 1 mM-EDTA, pH 7·4, or 1 mM-tris, pH 7·4, showed absolute stereospecificity for L-lactate.

Attempts to confirm the existence of the 'lactate racemase' of Huennekens *et al.* (1951*a*, *b*) were unsuccessful. Since liver and kidney particles had been found not to be absolutely specific for D-lactate, pigeon-breast-muscle mitochondria were used. Addition of 'racemase' preparations, made from rabbit-kidney cortex by the method of Huennekens *et al.* (1951*b*) did not enable D-lactate to act as a substrate (Table 1). Oxidation of

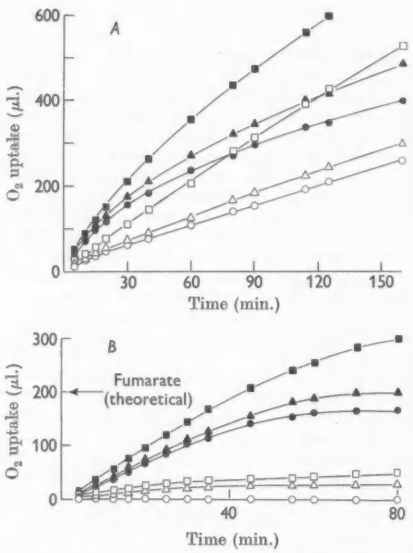

Fig. 1. *A*, Respiration of rabbit-kidney particles. Each vessel contained: phosphate, pH 7·5, 13 mM; $MgCl_2$, 3·6 mM; ATP, 1 mM; KCl, 42 mM; particles from 0·9 g. of cortex. Final vol., 3 ml. Where added, fumarate 1 mM and lactates 3·3 mM. Temp., 30°; equilibration, 6 min. The particles were prepared and twice washed in 0·125 M-KCl; N-NaOH was added during homogenization to maintain neutrality (cf. Huennekens *et al.* 1951*a*). *B*, Respiration of rat-liver mitochondria. Experimental conditions as in *A*, except that phosphate was 25 mM, lactates, where added, 13 mM, and temp. 38°. Each vessel contained mitochondria from 0·33 g. of tissue. In *A* and *B*: ○, no added substrate; △, L-lactate; □, D-lactate; ●, fumarate; ▲, fumarate + L-lactate; ■, fumarate + D-lactate.

Table 1. *Respiration of pigeon-breast-muscle mitochondria: failure of rabbit-kidney preparation to stimulate* D-*lactate oxidation*

Each vessel contained: phosphate, pH 7·5, 13 mM; $MgCl_2$, 3·3 mM; KCl, 110 mM; ATP, 1 mM; NAD, 0·15 mM; cytochrome *c*, 25 μM; fumarate, 0·33 mM; mitochondria from 1 g. of muscle. Lactates, when added, 14 mM. Final vol., 3 ml.; temp., 30°. Preparation from rabbit-kidney cortex was made by the procedure of Huennekens, Mahler & Nordmann (1951*b*); 0·5 ml. was added/vessel. Table shows O_2 uptake (μl.) in 30 min.

	Rabbit-kidney preparation	
	Heated*	Unheated
D-Lactate	16	37
L-Lactate	18	35
	96	270

* 10 min. at 100°.

Table 2. *Reduction of 2:6-dichlorophenol-indophenol by mitochondria in the presence of* D-*lactate*

Each Thunberg tube contained: mitochondria from 100 mg. of rabbit-kidney cortex; phosphate, pH 7·5, 17 mM; dye, 50 μM; KCl, 10 mM. Final vol., 3 ml.; temp., 37°. Reaction was started by the addition of dye and substrate from hollow stopper after 3 min. thermal equilibration. Air pressure was lowered to 0·3 atm. before incubation to avoid leakage due to increase in pressure on warming.

Additions: cyanide (mM)	1·7	1·7	33	33	1·7	1·7
D-Lactate (mM)	17	—	17	—	17	—
EDTA (mM)	—	—	—	—	1·0	1·0
Reduction time (min.)	3·3	>80	42	>80	60	>80

Table 3. *Inhibition of mitochondrial respiration by amytal*

Each vessel contained: phosphate, pH 7·5, 33 mM; tris, pH 7·6, 41 mM; $MgCl_2$, 7 mM; sucrose, 80 mM; substrates or KCl, 15 mM; glucose, 20 mM; ATP, 2 mM; excess of yeast hexokinase; mitochondria from 0·4 g. of rat liver. Sodium amytal or NaOH (final concn., 2 mM) added after 6 min. equilibration. Final vol., 3 ml.; temp., 30°. Rates of O_2 uptake were constant for 50 min. Rates in the absence of added substrate have been subtracted from the other rates.

Added substrate	O_2 uptake (μl./hr.) NaOH	O_2 uptake (μl./hr.) Amytal	Inhibition (%)
Succinate	420	438	−4
Pyruvate	93	2	98
D-Lactate	24	12	50
None	15	1	94

L-lactate, however, was enhanced by the addition of the kidney preparation. This was probably due to L-lactate dehydrogenase contained in it; Dr J. B. Chappell (personal communication) has found that addition of purified L-lactate dehydrogenase greatly stimulates the oxidation of L-lactate by pigeon-breast-muscle mitochondria prepared in potassium chloride–tris medium; with those prepared in sucrose–EDTA medium, however, addition of the dehydrogenase is not needed, and this has been confirmed in the present work.

Attempts were also made to prepare the 'racemase' from rat and rabbit liver. Despite variations in the method of preparation of 'racemase' and particles and in the conditions of the test, respiration in the presence of D-lactate and 'racemase' was never more than 10% greater than that in the presence of 'racemase' and absence of lactate, and always greatly less than that with L-lactate. The slight enhancement of activity in the presence of D-lactate was probably due to the glycollate oxidase (Kun *et al.* 1954) found to be present in the 'racemase' preparations. It is concluded that the existence of a mammalian α-hydroxy acid racemase must be regarded as doubtful.

The dye reduction (Thunberg tube) experiments of Mahler *et al.* (1953) have been repeated and extended. In agreement with these authors, NAD (0·15 mM) failed to stimulate indophenol reduction by rabbit-kidney mitochondria with D-lactate as substrate, whereas with L-lactate NAD was strongly stimulatory, especially after prolonged storage (up to 99 hr.) of the mitochondria at 0°. Metal-complex-forming agents inhibited the dye reduction (Table 2). Mahler *et al.* (1953) used 33 mM-cyanide in experiments similar to that in Table 2; that the strong inhibitory effect was not noticed was presumably due to the removal of volatile hydrocyanic acid by the unnecessarily efficient evacuation procedure.

Support for the hypothesis that NAD is not involved in the oxidation of D-lactate came from experiments on barbiturate inhibition. Jowett & Quastel (1937) found that tissue-slice respiration was inhibited by 5-ethyl-5-phenylbarbiturate (Luminal) when the substrate was glucose, L-lactate, pyruvate or L-glutamate, whereas with succinate oxygen uptake was actually enhanced. Ernster, Jalling, Löw & Lindberg (1955) found amytal to be the most effective compound, and concluded that only the oxidation of intramitochondrial reduced NAD, and of substrates generating this, was inhibited. Table 3 shows the effect of amytal on the oxidation of succinate, pyruvate and D-lactate by rat-liver mitochondria; similar results were obtained with rabbit-kidney mitochondria with which the D-lactate oxidation was more rapid. The apparent partial inhibition of D-lactate oxidation is compatible with complete amytal insensitivity for the step D-lactate → pyruvate, since the further oxidation of pyruvate presumably contributed to the oxygen uptake observed with D-lactate in absence of amytal.

The experiments with particulate preparations described above suggested that (*a*) the initial oxidation of D-lactate does not involve NAD, and (*b*) a metal is involved in this process. These conclusions are strongly supported by findings reported below and elsewhere (Tubbs, 1960*c*).

Experiments with soluble D-α-*hydroxy acid dehydrogenase*

Purification. Typical results of the purification procedure as described under Materials and Methods are summarized in Table 4. Extensive further purification could no doubt be achieved; the

Table 4. *Purification of enzyme from* 283 *g. of rabbit-kidney cortex*

For description of purification procedure and definition of unit of activity see Materials and Methods. Standard assay with 0·5 mM-cyanide.

Stage	Volume (ml.)	$\frac{E_{280\ m\mu}}{E_{260\ m\mu}}$	Protein (mg.)	Units	Units/mg.	Purification factor	Yield (%)
(1) Extract	126	0·86	690	2020	2·9	[1]	[100]
(2) Combined $(NH_4)_2SO_4$ ppts.	74·5	1·16	242	1790	7·4	2·5	89
(3) DEAE eluate (concentrated)	6·7	1·58	39·5	1610	40·8	13·9	80

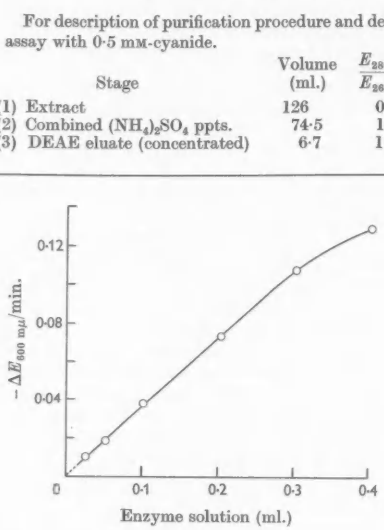

Fig. 2. Dependence of initial velocity on enzyme concentration. Enzyme (stage 2, Table 4) fully activated by storage for 2 days at 4°; the solution contained 4·3 mg. of protein/ml. Standard assay, without cyanide (see Materials and Methods).

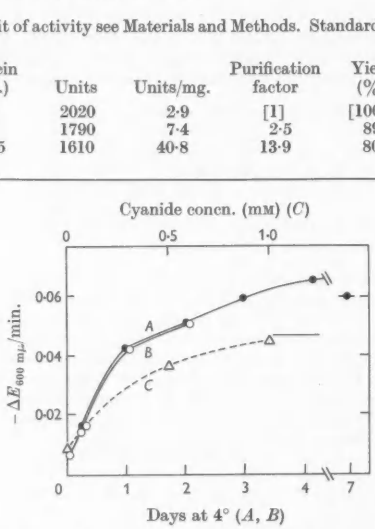

Fig. 4. *A*, *B*, Increase in activity of extract of dried mitochondria in 10 mM-tris, pH 7·8, during storage at 4°. Standard assay, except that pH was 8·1; cyanide absent; 2·8 mg. of protein/assay. *A* (●), Extract dialysed for 4 hr. against the above buffer immediately after preparation; *B* (○), extract not dialysed. *C*, Effect of cyanide in assay of a different extract from that in *A*, *B*. The extract (2·2 mg. of protein/assay) was tested at pH 8·1 1 hr. after preparation. The horizontal line shows the activity, without cyanide, after storage at pH 7·8 and 4° for 5 days.

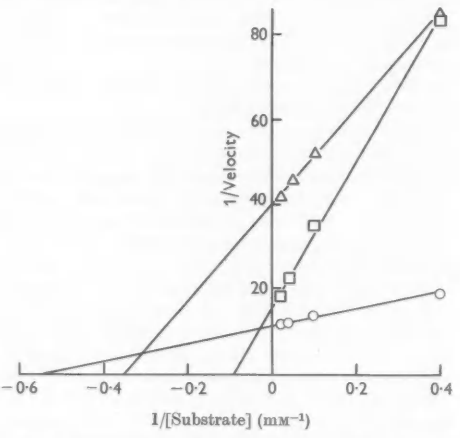

Fig. 3. Dependence of initial velocity on substrate concentration. Enzyme (stage 2, Table 4) fully activated by storage for 4 days at 4°. Standard assay, except that pH was 7·9 and substrate was varied; cyanide absent. Unit of velocity, $-E_{600\ m\mu}$/min. ○, D-Lactate; △, D-α-hydroxy-*n*-pentanoate; □, D-α-hydroxy-*n*-hexanoate.

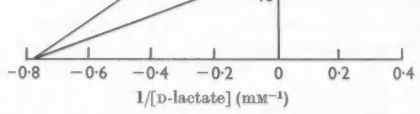

Fig. 5. Double-reciprocal plots showing that the increase in activity of the enzyme on storage was not due to change in K_m. Standard assay, except for variation in substrate concentration; 0·87 mg. of protein (stage 2, Table 4)/assay. Unit of velocity, $-E_{600\ m\mu}$/min. ○, 2 Days' storage at pH 7·8, 4°; ●, 7 days' storage.

limiting factor has been the small quantity of rabbit kidney available, for other tissues did not yield sufficiently active extracts by the method described.

Effect of enzyme and substrate concentrations. Progress curves were linear until about 50% of the indophenol had been reduced. The initial rate was proportional to the amount of added enzyme between 1 and 10 units (Fig. 2). The dependence of the initial velocity on substrate concentration is shown by double-reciprocal plots in Fig. 3. The Michaelis constant (K_m) for D-lactate has varied between 0·9 and 3·3 mM, but has usually been between 1 and 2 mM. No inhibition has been observed with excess of substrate up to at least 50 mM.

Activation on storage, or in the presence of cyanide. In preliminary work considerable difficulty was found in correlating results of experiments with the same extract on different days. This was because the enzyme undergoes 'spontaneous' activation. Fig. 4 (curves *A*, *B*) shows the rapid increase in enzyme activity which occurs on storing the crude extract at 4°; this increase is not due to a change in the affinity of the enzyme for the substrate (Fig. 5). Dialysis and partial purification of the enzyme, even through the DEAE stage, do not affect the activation process. No such 'spontaneous' activation in the cold seems to have been reported with other soluble oxidative enzymes, although superficially similar effects are shown by some hydrolytic enzymes (Gjessing, Emery & Clements, 1959).

Inclusion of dilute cyanide in the assay system resulted in immediate appearance of full activity (Fig. 4, curve *C*, and Fig. 6). EDTA, *o*-phenanthroline, oxalate, thiocyanate or azide did not activate; this renders unlikely the most obvious explanation for this effect of cyanide, namely the forming of complexes with contaminating metal ions. It may well be that the cyanide activation is related to the other effects of this compound on the enzyme (Tubbs, 1960*c*). Preincubation with substrate, a procedure effective with succinate dehydrogenase (Kearney, 1957), did not result in activation.

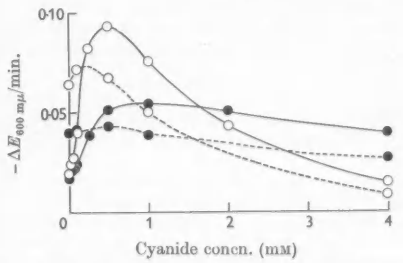

Fig. 6. Effect of cyanide in assay before and after storage of enzyme (stage 2, Table 4) at pH 7·9, 4°. Standard assay except for variation in pH; 0·65 mg. of protein/assay. ○, pH8·6; ●, pH 7·6; ——, enzyme stored 1 day; - - -, stored 8 days. (This preparation was unusual in that the activity under optimum conditions after 8 days' storage was less than that after 1 day.)

Substrate specificity. Table 5 shows the values of K_m and of velocity at infinite substrate concentration ($V_{max.}$) for various D-α-hydroxy acids, derived from double-reciprocal plots of results obtained in the indophenol assay. That the same enzyme was responsible for the oxidation of all these substrates was checked by mixed-substrate experiments, i.e. rates were determined with each substrate and D-lactate separately and also with equimolar mixtures of D-lactate with each other substrate. Insertion of the results into equation IV.69 of Dixon & Webb (1958) showed that only one dehydrogenase was involved, the calculated and observed velocities of the mixtures never differing by more than 12% and in most cases by much less.

The following hydroxy acids were not substrates: glycollate, L-lactate, D-α-hydroxy-β-methyl-*n*-pentanoate (both diastereoisomers), D-α-hydroxy-γ-methylpentanoate, L-tartrate, DL-mandelate, α-hydroxy*iso*butyrate, D-β-hydroxybutyrate and 3-phospho-D-glycerate. The limited evidence of these findings and those in Table 5 suggests that the structural features of a substrate include: (i) an α-hydroxy group and α-hydrogen, and D-configuration at the α-carbon atom; (ii) no branching at the β-carbon atom, although hydroxyl and carboxyl groups are permitted here; (iii) a side chain other than hydrogen, since glycollate is not a substrate.

Table 5 shows that K_m increases with chain length in the lactate homologues, and that $V_{max.}$ also varies with chain length. Other cases of

Table 5. *Substrate specificity of* D-α-*hydroxy acid dehydrogenase*

Standard assay except for D-malate and tartrates, when the buffer was phosphate, pH 6·6; with these substrates $V_{max.}$ is compared with that for D-lactate at pH 8·6, after allowance for the difference in the extinction of the indophenol at the two pH values.

	K_m (mM)	$V_{max.}$ (D-Lactate = 100)
D-Lactate	0·9–1·8	[100]
DL-α-Hydroxy-*n*-butyrate	2·9*	150
D-α-Hydroxy-*n*-pentanoate	2·9	29
D-α-Hydroxy-*n*-hexanoate	11·1	73
DL-α-Hydroxy-*n*-octanoate	12·5*	225
DL-Glycerate	10·5*	142
D-Malate	1·0	70
D-Tartrate	—	2–3
*meso*Tartrate	0·6	37

* Concn. of D-isomer.

variation of $V_{max.}$ with chain length have been discussed by Lumry (1959). Fig. 3 shows that the slopes of the double-reciprocal plots for these substrates, representing the ratio $K_m/V_{max.}$, increase with chain length, and Fig. 7 shows that this ratio is in fact directly proportional to the number of carbon atoms in the substrate. The interpretation of this curious finding is not at present clear.

Contrary to an earlier report (Tubbs & Greville, 1959), it has now been found that L-lactate is a weak competitive inhibitor of D-lactate oxidation, K_i being about four times the K_m for D-lactate. Assuming, as seems reasonable, that the L-enantiomorphs of other substrates also competitively inhibit, the K_m and $V_{max.}$ values shown in Table 5 will be too low in cases where the DL-compound was used. $K_m/V_{max.}$ (Fig. 7) will, however, be correct, as may be shown from the usual competitive-inhibition equation (Tubbs, 1961).

The one sample of D-glycerate tested gave a lower $V_{max.}$ than that with DL-glycerate, although K_m was the same with both. The reason for this is not known. A competitive inhibitor contaminating the D-glycerate would have decreased K_m as well as $V_{max.}$; a non-competitive inhibitor would have caused a non-linear double-reciprocal plot, and this was not observed.

The observation (Table 5) that *meso*tartrate is oxidized far more rapidly than D-tartrate, although the latter offers twice as many centres with the 'correct' configuration, may imply that the group not bound at the catalytic site causes inhibition when it has the D-configuration.

Influence of pH *and buffer composition on reaction rate.* Fig. 8 shows effects of different buffers on the rate of reduction of the indophenol by D-lactate and D-malate. The second negative charge on the latter substrate lowers the pH optimum by over 2 units. Pyrophosphate inhibits somewhat, perhaps by virtue of its chelating ability. A few measurements have been made of the variation of K_m and $V_{max.}$ with pH; K_m appears to increase, and $V_{max.}$ to decrease, on the acid side of the pH optimum (Fig. 9). Several factors influence the pH–activity curve when cyanide is present in the assay system [Fig. 6 and also Tubbs (1960*c*)]: (i) the activation of fresh enzyme by cyanide is more marked at the higher pH values; (ii) cyanide ion inhibits competitively, so inhibition by a given concentration of cyanide increases with pH, due to increasing dissociation; (iii) 'spontaneous' activation of the enzyme does not alter the K_m for substrate or the inhibition by cyanide, whereas it

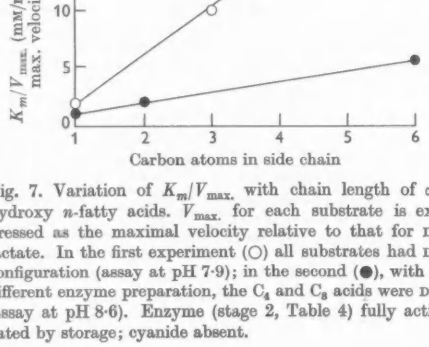

Fig. 7. Variation of $K_m/V_{max.}$ with chain length of α-hydroxy *n*-fatty acids. $V_{max.}$ for each substrate is expressed as the maximal velocity relative to that for D-lactate. In the first experiment (○) all substrates had D-configuration (assay at pH 7·9); in the second (●), with a different enzyme preparation, the C_4 and C_8 acids were DL (assay at pH 8·6). Enzyme (stage 2, Table 4) fully activated by storage; cyanide absent.

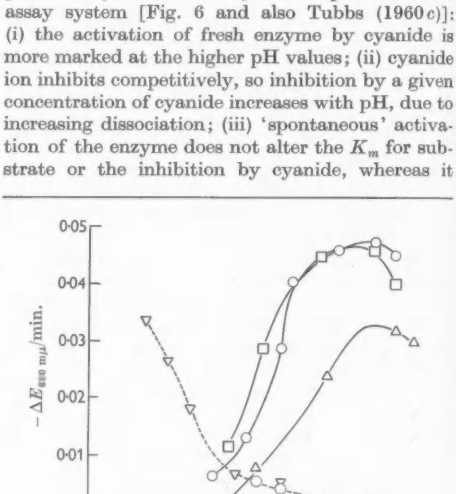

Fig. 8. Variation of enzyme activity with pH. Buffers 33 mM, substrates 25 mM. ○, Tris; □, glycylglycine; △, pyrophosphate; ▽, orthophosphate; —, D-lactate; - - -, D-malate. Enzyme (stage 2, Table 4) fully activated by storage; 0·43 mg. of protein/assay; cyanide absent. Allowances were made for change in extinction of the indophenol below pH 7.

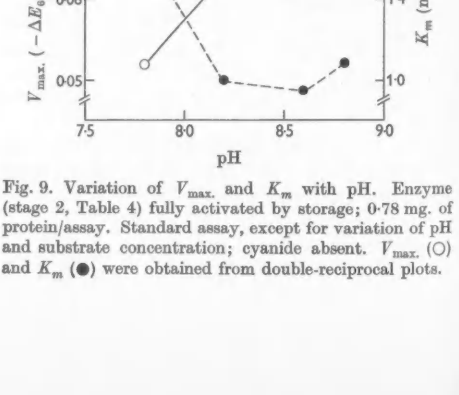

Fig. 9. Variation of $V_{max.}$ and K_m with pH. Enzyme (stage 2, Table 4) fully activated by storage; 0·78 mg. of protein/assay. Standard assay, except for variation of pH and substrate concentration; cyanide absent. $V_{max.}$ (○) and K_m (●) were obtained from double-reciprocal plots.

abolishes the acceleration by cyanide and so reduces the optimum concentration of this compound eventually to zero. The observed enzymic activity is thus dependent, in a complicated manner, on the 'age' of the enzyme, the pH, and the concentrations of substrate and of cyanide.

Reaction with various oxidants. In the presence of the dehydrogenase, the following substances have been found to be reduced by D-lactate: 2:6-dichlorophenol-indophenol, methylene blue, pyocyanine, phenazine methosulphate, cytochrome *c* and ferricyanide. There was no appreciable direct reaction with oxygen. No separation of indophenol- and cytochrome *c*-reducing activities was obtained by the partial purification of the enzyme, and, so far as tested, chelating agents were found to inhibit regardless of the acceptor employed.

The rate of reduction of the indophenol was little affected by change in dye concentration between 20 and 100 μM, under otherwise standard assay conditions; the apparent K_m for this dye is about 10 μM. Nevertheless, the indophenol assay does not measure the full activity of the enzyme, for the rate of dye reduction was increased by 35–40% when phenazine methosulphate (11 μM) was present as a mediator. The corresponding increase in the rate of reduction of cytochrome *c* (50 μM) was 440%. The optimum phenazine methosulphate concentration has not been determined, but with the above concentration the overall rate of dehydrogenation was the same whether the indophenol (38 μM) or cytochrome *c* (50 μM) was the final acceptor. NAD (0·15 mM) was not reduced by the enzyme and had no effect on the rate with the indophenol or cytochrome *c*; these observations were made with the salt-fractionated preparation.

The optimum pH has not yet been determined with acceptors other than the indophenol.

Stoicheiometry of D-lactate oxidation. The oxidation of D-lactate by oxygen has been followed with methylene blue as carrier. The partially purified extract used contained catalase. Table 6 shows that under these conditions the oxidation can be represented by the equations:

$$CH_3 \cdot CH(OH) \cdot CO_2^- + \text{methylene blue} \rightarrow CH_3 \cdot CO \cdot CO_2^- + \text{methylene blue} \cdot H_2, \quad \text{(i)}$$

$$\text{Methylene blue} \cdot H_2 + O_2 \rightarrow \text{methylene blue} + H_2O_2, \quad \text{(ii)}$$

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2. \quad \text{(iii)}$$

Overall:

$$CH_3 \cdot CH(OH) \cdot CO_2^- + \tfrac{1}{2}O_2 \rightarrow CH_3 \cdot CO \cdot CO_2^- + H_2O. \quad \text{(iv)}$$

The formation of pyruvate in a similar experiment to that in Table 6 has been confirmed by paper chromatography of the 2:4-dinitrophenylhydrazone, with butan-1-ol–aqueous ammonia as solvent (Cavallini, Frontali & Toschi, 1949). The enzymic reaction was stopped after 90 min., when 6·1 μmoles of oxygen had been taken up, by addition of trichloroacetic acid (to 3·3%, w/v), which precipitated nearly all the methylene blue. The main spot had R_F 0·35 (cf. Cavallini *et al.* 1949); a fainter spot at R_F 0·51 was due presumably to the second isomer of the dinitrophenylhydrazone (Stewart, 1953). Neither spot was obtained when either pyruvate or incubation was omitted.

In these experiments methylene blue was used in preference to phenazine methosulphate because the latter strongly inhibits catalase, and hydrogen peroxide decomposes pyruvate yielding acetate and carbon dioxide. With phenazine methosulphate it was therefore necessary to put alkali in the centre well of the manometer vessel before full oxygen uptake could be observed.

Possible flavoprotein nature of the enzyme. No stimulation of indophenol reduction by added FAD or FMN (10 μM) has been observed, even after repeated precipitation of the dehydrogenase with ammonium sulphate or partial inactivation by ultraviolet irradiation. Indeed, preincubation for 45 min. at 0° with a high concentration (1 mM) of FMN caused inhibition (50%), perhaps due to chelation, from which D-lactate protected. Preincubation with Mepacrine (5 mM) caused no inhibition; the corresponding yeast enzyme is inhibited by such treatment (Baudras, Iwatsubo & Labeyrie, 1960), but such inhibition is unreliable evidence of involvement of flavin (see Hemker & Hülsmann, 1960).

The absorption spectrum of the purest preparation obtained, determined in a Beckman recording spectrophotometer, showed no appreciable change in the 450 mμ region on addition of D-lactate, although yeast D-α-hydroxy acid dehydrogenase shows a flavin difference spectrum on reduction (Boeri, Cremona & Singer, 1960; Nygaard, 1960*a*). However, addition of D-lactate appeared to lower slightly an absorption peak at 405 mμ, and to

Table 6. *Stoicheiometry of oxidation of* D-*lactate by extract of rabbit-kidney mitochondria*

Each Warburg vessel contained, in a final vol. of 3 ml.: tris, pH 7·9, 42 mM; D-lactate, 25 mM; methylene blue, 2 mM; mitochondrial extract. Appropriate blanks and pyruvate standards were set up; these showed no O_2 uptake. After incubation for 77 min. at 30°, trichloroacetic acid was added and pyruvate determined by the method of Corwin (1959).

Enzyme (units)	O_2 uptake (μmoles)	Pyruvate formed (μmoles)	Pyruvate/O_2
15	2·20	4·7	2·14
30	3·95	8·5	2·15

increase the absorption below 380 mμ and between 425 and 350 mμ; a similar difference spectrum has been reported on reduction of chicken-kidney hypoxanthine dehydrogenase (Landon & Carter, 1960).

Analogy with other enzymes makes it probable that the present dehydrogenase is a flavoprotein; confirmation, however, must await further purification.

Distribution of the dehydrogenase. The reduction of indophenol by D-lactate is catalysed by mitochondria, and extracts of acetone-dried mitochondria, from kidneys of the rat, rabbit, sheep, ox and pig, and from livers of the rat, rabbit and ox. Of the tissues tested only pigeon breast muscle showed no activity. Hellerman, Reiss, Parmar, Wein & Lasser (1960) recently reported slow oxidation of D-malate by pig-heart extracts. Soluble preparations with activity adequate for detailed studies have so far been obtained only from rabbit kidneys; the rat-liver enzyme, however, is similar with respect to pH optimum, cyanide activation and inhibition by chelating agents.

DISCUSSION

Dehydrogenases resembling the one described in this paper have been found in yeast (Labeyrie, Slonimski & Naslin, 1959; Nygaard, 1959*a*, *b*, 1960*a*, *b*; Boeri *et al.* 1960), *Escherichia coli* (Haugaard, 1959) and other bacteria (de Ley & Schel, 1959; Snoswell, 1959; de Ley & Dochy, 1960; Molinari & Lara, 1960). In yeast (Nygaard, 1960*a*), *Acetobacter* (de Ley & Dochy, 1960) and perhaps in other organisms studied, D-lactate dehydrogenase activity is attached to insoluble material; the enzyme in *E. coli* seems to be associated with the cytochrome-containing particles described by Asnis, Vely & Glick (1956). No detailed study has been made of the intracellular distribution of the mammalian enzyme; however, it has been found to be absent from the soluble fraction of homogenates. It thus appears that NAD-independent D-lactate dehydrogenases are widely distributed and characteristically particle-bound; NAD-dependent enzymes are known, however, from *Chlorella* (Warburg, Gewitz & Völker, 1957) and lactobacilli (see Dennis & Kaplan, 1960).

There are no reports that D-lactate is a normal constituent of animal tissues, but it accumulates in *Chlorella* under anaerobic conditions (Warburg *et al.* 1957) and is present in considerable amounts in yeast (Singer, Kearney, Gregolin, Boeri & Rippa, 1960).

The metabolic significance of D-α-hydroxy acid dehydrogenase has still to be determined, but the presence of such an enzyme in widely different organisms suggests important functions. A few possibilities will be put forward, with special reference to the animal enzyme.

The putative succinate–glycine cycle (Shemin & Russell, 1953; Nemeth, Russell & Shemin, 1957) involves the conversion of δ-aminolaevulate into γδ-dioxovalerate, perhaps by transamination (Braunstein, 1960, p. 184). This keto-aldehyde is transformed into D-α-hydroxyglutarate by glyoxalases from various animal tissues (for references see Dalgleish, Johnson, Long & Tyler, 1952, p. 1114). It has not yet been possible to test the last-named substance with D-α-hydroxy acid dehydrogenase, but in view of the results with D-malate it is likely to be a substrate; if so, the enzyme could provide the link which allows completion of the cycle.

It has been suggested (Shemin, 1955; Gibson, Laver & Neuberger, 1958) that the reactions of the succinate–glycine cycle may be extended to form an acetate–glycine cycle; this has been formulated by Elliott (1959). Here, aminoacetone, derived from glycine and acetyl-coenzyme A or from threonine (cf. Urata & Granick, 1961), is deaminated yielding methylglyoxal, which is converted into D-lactate by glyoxalase. The next step offers a role for D-α-hydroxy acid dehydrogenase, and oxidative decarboxylation of the pyruvate formed completes the cycle. As to whether this cycle takes place in animal tissues, it may be noted that aminoacetone is formed from acetyl-coenzyme A and glycine by particles from avian erythrocytes (Gibson *et al.* 1958) and from guinea-pig liver (Urata & Granick, 1961), and is converted into carbon dioxide in the rat (Nemeth *et al.* 1957) and to methylglyoxal by an enzyme from ox plasma (Elliott, 1960).

D-Glycerate was oxidized by the present dehydrogenase, and a positive naphthalene-1:3-diol test (Dickens & Williamson, 1958) indicated that the product was hydroxypyruvate. The enzyme may therefore be involved in the biosynthesis of L-serine, since one possible route for the formation of this amino acid from carbohydrate in animals is via D-glycerate and hydroxypyruvate, the latter yielding serine through the action of a reversible alanine–hydroxypyruvate transaminase (Sallach, 1955). In the opposite direction, hydroxypyruvate and D-glycerate are both converted into carbohydrate by animal tissues (Dickens & Williamson, 1959, 1960); hence if the action of D-α-hydroxy acid dehydrogenase on D-glycerate is reversible, the enzyme may also be involved in glyconeogenesis. It may be remarked that the yeast enzyme can reduce pyruvate to D-lactate (Labeyrie, Naslin, Curdel & Wurmser, 1960) and that the redox potential of the glycerate–hydroxypyruvate system is close to that of lactate–pyruvate (Zelitch, 1955).

Finally, the dehydrogenase may possibly play some part in the metabolism of the long-chain

D-α-hydroxy fatty acids found in cerebrosides (Skipski, Arfin & Rappoport, 1959; Kishimoto & Radin, 1959).

SUMMARY

1. Liver and kidney mitochondria oxidize D-lactate much faster than L-lactate, whereas pigeon-breast-muscle mitochondria oxidize only the L-isomer. This is in agreement with Huennekens *et al.* (1951*a*, *b*), but their finding that liver and kidney contain a 'racemase' enabling pigeon-muscle particles to oxidize D-lactate could not be confirmed.

2. Reduction of dyes by kidney mitochondria in the presence of D-lactate is inhibited by metal-complex-forming agents and not stimulated by nicotinamide–adenine dinucleotide.

3. A soluble D-α-hydroxy acid dehydrogenase has been extracted from rabbit-kidney-cortex mitochondria and partially purified.

4. The enzyme, which occurs in liver and kidney in several animal species, catalyses oxidation of lactate and straight-chain homologues, glycerate, malate and tartrate; it was specific for the D-isomer whenever this could be tested. The product of D-lactate oxidation is pyruvate.

5. With lactate and homologues, $K_m/V_{max.}$ is proportional to chain length.

6. Electron acceptors include various dyes, ferricyanide and cytochrome *c*, but not oxygen. Nicotinamide–adenine dinucleotide is not needed.

7. The enzyme when extracted has low activity, but undergoes activation during storage at 4°. Inclusion of cyanide in the assay system causes immediate full activation.

8. Possible metabolic roles of the enzyme are discussed.

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Thymidine Diphosphate Mannose and Thymidine Diphosphate Rhamnose in *Streptomyces griseus*

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Streptomyces griseus is grown in large amounts for the commercial production of streptomycin. Our interest in the mechanism of biosynthesis of the antibiotic led to a study of nucleotides in this organism, as it seemed possible that the carbohydrate residues in streptomycin, *N*-methyl-L-glucosamine and streptose, would occur in combination with nucleotides at some stage in the biosynthetic route.

Although nucleotides containing streptose or *N*-methyl-L-glucosamine have not yet been found in the complex mixture obtained from the microorganism, several new nucleotides were detected. Two of these have been identified as thymidine diphosphate mannose and thymidine diphosphate rhamnose. The isolation and structure of these compounds is described, and their possible function as streptomycin precursors is discussed. A mechanism for the biosynthesis of rhamnose through thymidine derivatives is suggested. A preliminary account of some of this work has been published (Baddiley & Blumsom, 1960).

MATERIALS AND METHODS

Abbreviations. TDP-mannose, thymidine diphosphate mannose; TDP-rhamnose, thymidine diphosphate rhamnose; TDP-glucose, thymidine diphosphate glucose.

Thymidine was purchased from the California Corp. for Biochemical Research, Los Angeles 63. Thymidine 5′-phosphate was kindly supplied by Dr A. M. Michelson. Uridine diphosphate glucose was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Guanosine diphosphate mannose and uridine diphosphate *N*-acetylglucosamine were kindly supplied by Dr E. Cabib. DL-Glyceric acid was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. D-Glyceric acid was obtained by hydrolysis of its 3-phosphate with a prostate-phosphatase preparation. DPNH was purchased from C. F. Boehringer,

Mannheim. Rabbit-muscle enzymes were prepared by Racker's (1947) method. Crude yeast hexokinase was a gift from Sigma Chemical Co., St Louis, Mo.

Quantitative analysis. Phosphate was determined by the method of Fiske & Subbarow (1925), and reducing sugars were determined by the method of Park & Johnson (1949). Rhamnose was also determined according to Dische & Shettles (1948). Quantitative determination of thymine, thymidine and thymidine 5′-phosphate was based on molar extinction values at the appropriate wavelength of maximum absorption at known pH values.

Paper chromatography. Whatman no. 1 paper, previously washed with 2N-acetic acid and then water, was used with ascending or descending solvents.

Sugars were examined in butan-1-ol–pyridine–water (6:4:3) (Jeanes, Wise & Dimler, 1951) or in phenol–water (9:1) (Partridge, 1948). Spots were detected with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) or with the phloroglucinol–hydrochloric acid reagent (Borenfreund & Dische, 1957). Polyols were converted in part into anhydro compounds and examined in propan-1-ol–ammonia (sp.gr. 0·88)–water (6:3:1) with the periodate–Schiff reagents (Baddiley, Buchanan & Carss, 1957*b*).

Pyrimidines and nucleotides were examined in butan-1-ol–water (Markham & Smith, 1949), propan-2-ol–ammonia (sp.gr. 0·88)–water (85:1·3:15) (Hershey, Dixon & Chase, 1953), propan-2-ol–conc. hydrochloric acid (171:41) (Wyatt, 1951), or two ethanol–ammonium acetate mixtures (Paladini & Leloir, 1952). The position of these compounds was demonstrated by observation in ultraviolet light. Nucleotides were also located with perchloric acid–molybdate reagent (Hanes & Isherwood, 1949).

Ionophoresis on paper was carried out with apparatus similar to that described by Markham & Smith (1952). Whatman no. 1 paper moistened with 0·05M-sodium borate solution (pH 9·2) was used for sugars (Consden & Stanier, 1952), a potential of 7·5 v/cm. being applied for 6 hr. The sugars were detected with alkaline silver nitrate by the modified technique of Frahn & Mills (1959).

Isolation of nucleotides

An ethanolic extract of *Streptomyces griseus* was kindly supplied by Dr I. L. S. Mitchell of Glaxo Laboratories Ltd. It had been prepared in the following manner.

Cells were harvested and treated immediately with an equal volume of ethanol. The suspension was boiled with stirring and kept at the boil for a further 2 min., then cooled and filtered. The residue was heated with two lots of ethanol as before and the extracts were combined and adjusted to pH 7·0.

The ethanol extract (5 l.) was passed through a column (50 cm. × 4 cm.) of Dowex 1X2 resin (Cl^- form). The column was washed with water, then with 0·005N-hydrochloric acid until the extinction of the washings at 260 mμ was negligible. Nucleotides were eluted with a solution that was 0·01N with respect to hydrochloric acid and 0·18M with respect to lithium chloride. The eluate was neutralized with lithium hydroxide, evaporated *in vacuo* to about 70 ml. and poured with stirring into acetone–ethanol (4:1) (2 l.). The light-brown precipitate was collected, washed with acetone–ethanol (4:1) to remove traces of lithium chloride, dried *in vacuo* and stored at −20°.

A solution of the nucleotide mixture (about 1700 μmoles, calculated as uridine from its absorption at 260 mμ) in water (1 l.) was passed through a column (100 cm. × 2 cm.) of Dowex 1X2 (200–400 mesh) resin (Cl^- form). Elution was achieved with a linearly increasing concentration of lithium chloride at constant acid concentration in an apparatus similar to that described by Pontis & Blumsom (1958). The reservoir contained 4 l. of a solution which was 0·16M with respect to lithium chloride and 0·01N with respect to hydrochloric acid; the mixing chamber contained 0·01N-hydrochloric acid (4 l.). A flow rate of about 1·5 ml./min. was maintained and 10-ml. fractions were collected automatically. Compounds that absorbed ultraviolet light were detected, and their approximate amount was recorded automatically as they emerged from the column, by a combined ultraviolet light source, photocell, amplifier and recorder (Gilson Medical Electronics, Madison, Wis.). Elution was continued until the concentration of lithium chloride in the mixing chamber rose to 0·14M. At this point the reservoir was replaced by one containing 2 l. of a solution which was 0·18M with respect to lithium chloride and 0·01N with respect to hydrochloric acid, and the solution in the mixing chamber was changed to 2 l. of a solution which was 0·14M with respect to lithium chloride and 0·01N with respect to hydrochloric acid.

Fractions corresponding to ultraviolet-absorption peaks were combined, adjusted to pH 7·0 with lithium hydroxide solution and freeze-dried. Lithium chloride was extracted with an acetone–ethanol mixture as before and the dried preparations were kept at −20°.

Preparation of hydrolysates

Hexoses. The following procedure was adopted for the preparation of hexoses liberated by acid hydrolysis of the pyrimidine nucleotides from the organism. Appropriate nucleotide fractions were hydrolysed at 100° in 0·01N-sulphuric acid for 20 min. The resulting solutions were passed through small columns of Dowex 50 (H^+) and Amberlite IR-4B(OH^-) resins and the eluates were evaporated *in vacuo*. The residues were examined by paper chromatography.

Bases. Thymine was obtained from appropriate fractions in the following manner. The material was heated at 100° in 6N-hydrochloric acid for 8 hr. Most of the acid was removed by repeated evaporation with water, and an aqueous solution was then passed through columns of resins as for hexoses. The residue was examined spectroscopically and by paper chromatography.

Synthesis of thymidine diphosphate mannose

Calcium thymidine 5′-phosphate (300 mg.) was converted into the ammonium salt by passing its solution through a column of Dowex 50 (H^+) resin, neutralizing with ammonia and evaporating the resulting solution to dryness *in vacuo*. To the residue was added formamide (7·5 ml.), 2N-ammonia solution (2·0 ml.) and a solution of di*cyclo*hexylcarbodi-imide (900 mg.) in *tert.*-butyl alcohol (6 ml.). The mixture was heated for 10 hr. at 80° and di*cyclo*hexylurea was removed by filtration. The *tert.*-butyl alcohol was removed from the filtrate under reduced pressure and the aqueous formamide solution was shaken with four lots of ether. Solvent was evaporated (oil pump), acetone (55 ml.) and ether (180 ml.) were added, and the mixture was kept in a refrigerator overnight. Solvent was

decanted from the semi-solid precipitate, which was dried *in vacuo*, then dissolved in water (25 ml.) and freeze-dried.

The phosphoramidate was homogeneous on paper chromatography in the propan-1-ol–ammonia solvent and had R_{TMP} 1·86. It was detected by observation in ultraviolet light and with the chlorine–benzidine reagents (Reindel & Hoppe, 1954). The yield, calculated from the absorption of a sample at 268 mμ, was 580 μmoles.

Barium mannose 1-phosphate (Colowick, 1938) was converted into the tri-*n*-octylammonium salt by a procedure similar to that described by Michelson & Todd (1956) for glucose 1-phosphate. A solution of the tri-*n*-octylammonium salt (2·5 m-moles) in dry pyridine (25 ml.) was added to a solution of the phosphoramidate (580 μ-moles) in dry pyridine (10 ml.), and the mixture was shaken under anhydrous conditions for 5 days at 28°. Paper chromatography (ethanol–ammonium acetate, pH 3·8) indicated that the mannose nucleotide was formed in about 65% yield.

Most of the pyridine was evaporated *in vacuo*, water (25 ml.) was added, and the solution was shaken with three lots of ether. After aeration, products were adsorbed on a column (100 cm. × 1 cm.) of Dowex 1X2 (200–400 mesh; Cl^- form). Elution was carried out with a linear gradient, with in the mixing flask a solution (2 l.) which was 0·1 M with respect to lithium chloride and 0·01 N with respect to hydrochloric acid, and in the reservoir a solution (2 l.) which was 0·18 M with respect to lithium chloride and 0·01 N with respect to hydrochloric acid. Fractions (10 ml.) were collected automatically at a flow rate of 1·5 ml./min. The mannose nucleotide emerged after about 2 l. of solution had been passed through the column. Appropriate fractions were combined, the pH was adjusted to 7 with lithium hydroxide solution and water was removed by evaporation *in vacuo* to a final volume of 5 ml. A mixture (1 l.) of acetone–ethanol (4:1) was added and, after keeping at −14° overnight, the precipitated *lithium salt of TDP-mannose* was collected, washed with the acetone–ethanol mixture until free from chloride ions, dried (phosphorus pentoxide) and kept under anhydrous conditions at 0° (Found: P, 9·2; thymine, 17·9. $C_{16}H_{24}O_{16}N_2P_2Li_2,4H_2O$ requires P, 9·5; thymine, 17·7%).

RESULTS

Several of the nucleotides corresponding to absorption peaks in the ion-exchange fractionation of the mixture from the organism were tentatively identified from their position in the elution pattern, ultraviolet absorption and behaviour on paper chromatography. Thus, the compound corresponding to peak VII is probably uridine diphosphate glucose and that corresponding to peak IX is uridine 5′-pyrophosphate. The intermediate peak VIII represented thymidine derivatives and corresponds to the material used in most of the work described here. R_F values for these nucleotides and several known compounds are given in Table 1.

Material corresponding to peak VIII was usually light yellow. The coloured impurity was removed by chromatography in the ethanol–ammonium acetate (pH 3·8) solvent. Complete separation of

Table 1. *Paper chromatography of nucleotides from* Streptomyces griseus *and standard compounds*

	$R_{adenosine}$ in ethanol–ammonium acetate–water at	
	pH 3·8	pH 7·5
Uridine 5′-phosphate	0·72	0·33
Uridine 5′-pyrophosphate	0·38	0·18
Guanosine diphosphate mannose	0·23	0·30
Uridine diphosphate glucose	0·40	0·41
Uridine diphosphate *N*-acetylglucosamine	0·54	0·53
Thymidine 5′-phosphate	0·77	0·50
Compound corresponding to peak VII	0·40	0·39
Compounds corresponding to peak VIII	0·53, 0·55	0·50, 0·35
Compound corresponding to peak IX	0·38	0·18
Nucleotide after treatment of VIII with 0·1 N-HCl at 100° for 15 min.	0·76	0·50

Table 2. *Paper chromatography and ionophoresis of hexoses from thymidine derivatives and standard compounds*

	R_{ribose} in		Migration (cm.) on ionophoresis
	Butanol–pyridine–water	Phenol–water	
Fructose	0·85	0·65	1·15
Glucose	0·77	0·47	1·30
Mannose	0·85	0·55	0·89
Galactose	0·70	0·56	1·18
Rhamnose	1·09	1·05	0·66
Sugars from thymidine compounds	0·85, 1·07	0·54, 1·04	0·89, 0·65

the two nucleotide components in this fraction was only achieved after prolonged (40 hr.) paper chromatography.

Identification of hexoses

The hexoses liberated on mild acid hydrolysis of the thymidine compounds were examined on paper with butanol–pyridine–water or phenol–water solvent systems and by ionophoresis. From results given in Table 2 it was concluded that the hexose components were rhamnose and mannose.

The sugar mixture was reduced with sodium borohydride during 24 hr. at room temperature (Abdel-Akher, Hamilton & Smith, 1951). The salt-free products were heated for 2 hr. at 100° in 5N-hydrochloric acid, whereupon a mixture of hexitols and anhydro compounds was obtained. After evaporation of acid these were examined by paper chromatography in the propan-1-ol–ammonia solvent and detected with the periodate–Schiff reagents.

The ratio mannose:rhamnose in the mixture was calculated from the value for total reducing sugar (Park & Johnson, 1949) and the value for rhamnose (Dische & Shettles, 1948). The former method is equally sensitive to both sugars, whereas the latter method is insensitive to mannose. The ratio observed was 4:1.

Identification of base

The ultraviolet spectra at pH 7 and 12 of the nucleotide corresponding to peak VIII suggested that the base residue was entirely thymine. This was confirmed by chromatographic examination of the products of hydrolysis in 6N-hydrochloric acid. Thymine was identified by comparison with authentic bases in several solvent systems (Table 3).

Less vigorous hydrolysis (0·1N-hydrochloric acid for 15 min. at 100°) gave one nucleotide. This was examined by evaporation of acid, neutralization with ammonia and chromatography in the ethanol–ammonium acetate solvents (Table 1). The nucleotide was indistinguishable from authentic thymidine 5′-phosphate.

Quantitative analyses

The ratio thymidine:total phosphate:labile phosphate:hexose was 1·04:2·0:0·96:1·06 for the mixture of the two nucleotides. Total phosphate was taken as 2·0 and labile phosphate was determined by hydrolysis for 15 min. at 100° in N-sulphuric acid.

Identification of thymidine and its 5′-phosphate

Hydrolysis of the thymine nucleotide mixture in 0·1N-hydrochloric acid at 100° for 15 min. gave a nucleotide (see Table 1) indistinguishable from thymidine 5′-phosphate. It was isolated by absorption on a small column of Amberlite IR-4B (OH^-) resin, elution with 5N-ammonia solution and evaporation to dryness.

The location of the phosphate residue in this nucleotide was confirmed by studies with *Crotalus adamanteus* venom. A solution of the nucleotide in water (0·2 ml.) and 0·1M-barbitone buffer, pH 9·0 (0·4 ml.), was mixed with a solution (0·3 ml.) of the venom (5 mg./ml.) and kept for 16 hr. at 37°. After removal of protein with trichloroacetic acid (final concentration, 3%) followed by centrifuging, most of the buffer was precipitated by evaporation *in vacuo*. Paper chromatography in *iso*propanol–ammonia and rechromatography in butan-1-ol–water yielded thymidine, identical with authentic material (see Table 3). The nucleoside did not react with the periodate–Schiff reagents (Buchanan, Dekker & Long, 1950; Baddiley, Buchanan, Handschumacher & Prescott, 1956) but gave a positive reaction for deoxy-sugars with the cysteine–sulphuric acid reagent (Buchanan, 1951).

Analysis of a sample of the thymidine from a paper chromatogram by the cysteine–sulphuric acid method for deoxy-pentoses (Stumpf, 1947; Manson & Lampen, 1951) gave a ratio thymine:deoxyribose as 1·03:1·0 (deoxyribose taken as 1·0).

Stability of mannose and rhamnose nucleotides towards ammonia

Samples of pure TDP-rhamnose, TDP-mannose, uridine diphosphate glucose and guanosine diphos-

Table 3. *Paper chromatography of base and nucleoside compared with authentic compounds*

	$R_{adenine}$ in		
	Butan-1-ol–water	Propan-2-ol–ammonia	Propan-2-ol–HCl
Guanine	0·33	0·41	0·63
Uracil	0·78	0·97	1·88
Thymine	1·19	1·33	2·17
Uridine	0·38	0·79	1·83
Thymidine	1·12	1·46	2·31
Base from VIII	1·19	1·32	2·17
Nucleoside from VIII	1·12	1·44	2·29

phate mannose were dissolved in N-ammonia solution and kept for 18 hr. at 27°. The samples were then examined by paper chromatography in the ethanol–ammonium acetate (pH 3·8) solvent mixture, products being detected by examination in ultraviolet light and with the perchloric acid–molybdate reagent. Semi-quantitative information was obtained spectroscopically.

About 98% of the uridine diphosphate glucose and all the TDP-rhamnose had been converted into hexose cyclic phosphate and nucleoside monophosphates under the conditions employed, whereas guanosine diphosphate mannose and TDP-mannose were unaffected. The rhamnose cyclic phosphate (R_{TMP} 0·84) was not studied further.

Oxidation of thymidine diphosphate mannose to D-glyceric acid

Uridine diphosphate glucose and TDP-mannose were oxidized with periodate, then bromine, and glyceric acid was separated from the hydrolysed products. The following procedure and quantitative determinations are based on studies with the mannose-containing nucleotide; the procedure for the glucose compound was closely comparable. Glyceric acid 3-phosphate arising from the ribose phosphate residue in uridine diphosphate glucose would not have been hydrolysed under the acidic conditions used at one stage in this method, and thus would not contribute to the value found for D-glyceric acid.

The nucleotide (2·5 μmoles) was mixed with 0·13M-sodium periodate (0·2 ml.) and, after 48 hr. at room temperature in the dark, excess of periodate was destroyed by the addition of ethylene glycol (0·01 ml.). The solution was kept for a further 48 hr. at room temperature then mixed with an excess of bromine in a buffer consisting of barium hydroxide (189 mg.) and benzoic acid (146 mg.) in water (35 ml.) at pH 5·5. After 60 hr. at room temperature in the dark, excess of bromine was removed by aeration and the solution was concentrated to 0·5 ml. *in vacuo*. Precipitated benzoic acid was removed by centrifuging and the solution was passed through a column (5 cm. × 0·3 cm.) of Amberlite IR-120 (H^+) resin to remove barium ions, then evaporated to dryness at room temperature. The residue was hydrolysed with 5N-hydrochloric acid for 5 hr. at 100°. Acid was removed by repeated evaporation with water and the pH was adjusted to a value above 11 with sodium hydroxide. This ensured hydrolysis of the bimolecular lactone of glyceric acid, which could be demonstrated chromatographically in the acid hydrolysate. Glyceric acid was separated by paper chromatography in propan-1-ol–ammonia and eluted from the paper with water. The purple colour it gave with the periodate–Schiff reagents developed rapidly. The eluate, and an eluate from a corresponding control area of paper, were evaporated to dryness and the residues dissolved in water (0·1 ml.).

A sample (5 μl.) of the glyceric acid solution was used for total glyceric acid determination. This was carried out by periodate oxidation (Dixon & Lipkin, 1954) during 60 min. at room temperature. Under these conditions 1 mol.prop. of periodate was consumed for each mol.prop. of glyceric acid. Further consumption of periodate was very slow. The concentration of glyceric acid determined by this method was 2·0 μmoles/ml.

Enzymic determination of D-glyceric acid was carried out according to the procedure described by Archibald, Baddiley & Buchanan (1961). Phosphorylation and reduction were combined in a single operation by mixing a sample (0·08 ml.) of the glyceric acid solution with a solution containing the following: 0·01M-phosphate buffer, pH 7·3 (3·0 ml.); 0·1M-magnesium sulphate (0·05 ml.); 0·2M-adenosine triphosphate (0·05 ml.); rabbit-muscle enzymes (0·05 ml. of solution containing 40 mg./ml.); hexokinase (0·05 ml. of solution containing 80 mg./ml.), and reduced diphosphopyridine nucleotide (0·07 ml. of solution containing 20 mg./ml.). Oxidation of the pyridine nucleotide was observed spectrophotometrically at 37° during 60 min. The concentration of D-glyceric acid was 2·1 μmoles/ml., in close agreement with the value found by the chemical method. L-Glyceric acid was not reduced in this system.

DISCUSSION

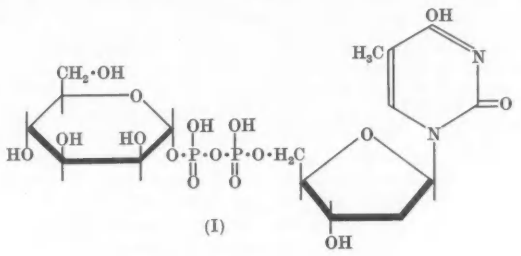
Particular attention was directed towards a fraction which was eluted between uridine diphosphate glucose and uridine diphosphate, and which had an absorption spectrum closely similar to that of thymidine and its derivatives. Although this fraction contained two nucleotides, much of the preliminary degradative work was carried out with this rather than with the individual nucleotides. The mixture was hydrolysed at pH 1 for 15 min. at 100°. These hydrolysis conditions would be expected to release sugars from nucleoside diphosphate sugar compounds. Two sugars with R_{ribose} values corresponding to mannose and rhamnose were detected, and their identification was confirmed by paper electrophoresis in a borate buffer (pH 9·2). An alternative method for the identification of small amounts of sugars was applied successfully to this mixture. The sugars were reduced with sodium borohydride to the corresponding sugar alcohols, and these were readily identified by heating with mineral acid and examining the reaction mixture on paper chromatograms. It is known (Baddiley, Buchanan & Carss, 1957*b*) that

pentitols and hexitols treated in this way give anhydropolyols or mixtures of anhydropolyols that can be identified chromatographically. The position, colour and relative intensity of the spots corresponding to anhydropolyols obtained from the sugar alcohol mixture were identical with that given by a mixture (4:1) of mannitol and rhamnitol which had been treated similarly. It was shown from studies on the separated nucleotides that rhamnose was associated with the faster-moving nucleotide and mannose with the slower.

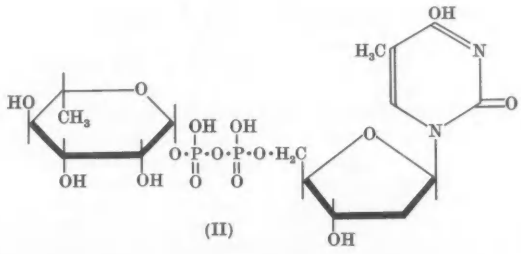
Although the ultraviolet-absorption spectrum of the nucleotide mixture suggested that both components were thymidine derivatives, the close similarity between the spectra of thymidine phosphate and uridine phosphate made it necessary to use other methods for the identification of the base. When the mixture was hydrolysed for 8 hr. at 100° in 6N-hydrochloric acid the only base formed was thymine. Moreover, hydrolysis at pH 1, followed by treatment with *Crotalus adamanteus* venom at pH 9, liberated thymidine. It gave the typical reaction for deoxynucleosides with the cysteine–sulphuric acid spray reagents, and the negative periodate–Schiff reaction on paper indicated the absence of an α-glycol system.

These observations and the quantitative analyses indicate that in the two nucleotides thymidine is joined at the 5′-position through a pyrophosphate residue to either mannose or rhamnose as shown in structures (I) and (II) respectively. The ready liberation of thymidine by the action of *C. adamanteus* venom on an acid hydrolysate indicates that a pyrophosphate residue is at the 5′-position, since it is known (Heppel & Hilmoe, 1951) that this venom contains a powerful 5′-nucleotidase, but has little action on nucleoside 3′-phosphates. The absence of reducing properties before hydrolysis, and the ready liberation of mannose and rhamnose by gentle acid hydrolysis, support the normal structure for this class of nucleotides in which the pyrophosphate group is attached to the 1-position of the hexose.

The stereochemical configuration of the mannose in TDP-mannose was established by a technique developed in connexion with other work (Archibald *et al.* 1961) and not previously applied to sugar phosphates. The nucleotide was first oxidized with periodate and the resulting dialdehyde was then oxidized with bromine water to a dicarboxylic acid (see Fig. 1). Acid hydrolysis gave glyceric acid together with glyoxylic acid and thymidine 5′-phosphate. The stereochemical configuration of the glyceric acid was established by enzymic phosphorylation followed by quantitative determination of D-glyceric acid 3-phosphate through reduction with reduced diphosphopyridine nucleotide in the presence of enzymes from rabbit muscle (cf. Baddiley, Buchanan & Carss, 1957*a*). All the glyceric acid formed in this way from TDP-mannose had the D-configuration, and it follows that the hexose in this nucleotide is D-mannose. Although this procedure can be applied to quite small samples of sugar phosphates or nucleotides,



(I)



(II)

the yield of glyceric acid is rather low. This probably arises through the complexity of the final oxidation mixture and the difficulty experienced in removing ions and nucleotide fragments before determining the glyceric acid. The acidic conditions that follow the oxidation sometimes cause partial conversion of the resulting glyceric acid into a dimeric lactone. This compound, which gives an amide with an R_F slightly higher than that of glyceric acid in propan-1-ol–ammonia, is readily converted into glyceric acid in alkali. It is reasonable to assume that the TDP-rhamnose is a derivative of L-rhamnose, this being the only natural form of rhamnose observed so far.

The configuration and conformation of the substituent group at the 1-position in the mannose and rhamnose residues of these nucleotides has been determined by studying their behaviour towards dilute ammonia in comparison with that of uridine diphosphate glucose. It is known that nucleoside diphosphate sugar coenzymes, like phosphodiesters, are unstable towards dilute alkali if they possess a hydroxyl group in suitable stereochemical proximity to the pyrophosphate group. Hydro-

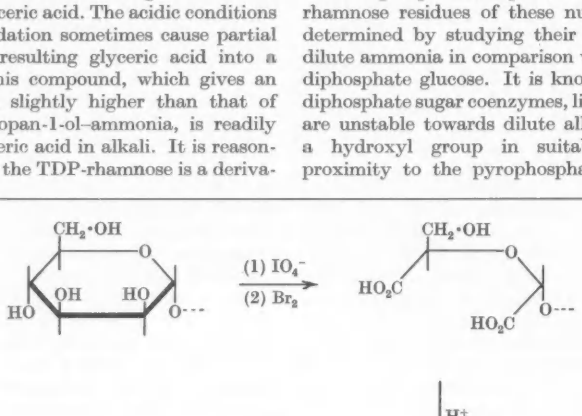


Fig. 1. Degradation of TDP-mannose to D-glyceric acid.

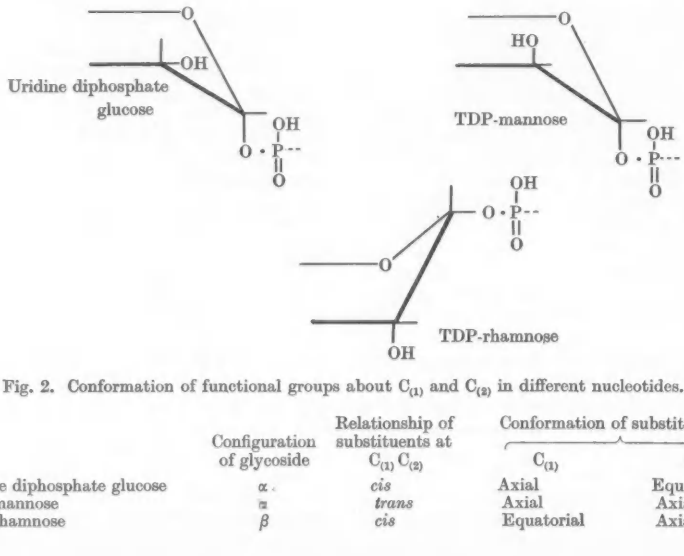


Fig. 2. Conformation of functional groups about $C_{(1)}$ and $C_{(2)}$ in different nucleotides.

	Configuration of glycoside	Relationship of substituents at $C_{(1)}$ $C_{(2)}$	Conformation of substituent at $C_{(1)}$	Conformation of substituent at $C_{(2)}$
Uridine diphosphate glucose	α	*cis*	Axial	Equatorial
TDP-mannose	α	*trans*	Axial	Axial
TDP-rhamnose	β	*cis*	Equatorial	Axial

lysis proceeds through intermediate formation of cyclic phosphates, and under suitable conditions the cyclic phosphate may be a principal hydrolysis product. Thus, the ready decomposition of uridine diphosphate glucose to glucose 1:2-cyclic phosphate in the presence of dilute ammonia at room temperature (Paladini & Leloir, 1952) supports the conclusion from other evidence that this nucleotide is a derivative of α-glucose 1-phosphate. In this case the *cis* relationship of the functional groups at the 1- and 2-positions greatly facilitates cyclic phosphate formation (Fig. 2).

Guanosine diphosphate mannose differs in that it is quite stable towards dilute ammonia (Cabib & Leloir, 1954), because of the *trans* relationship of the functional groups at the 1- and 2-positions in α-D-mannose 1-phosphate (Fig. 2) (Buchanan, Lynch, Benson, Bradley & Calvin, 1953). These groups in derivatives of α-D-mannose will both adopt the axial conformation and cyclization would be extremely difficult. Under conditions which ensured complete decomposition of uridine diphosphate glucose, both TDP-mannose and guanosine diphosphate mannose were unaffected; it follows that TDP-mannose must be an α-D-mannose derivative with the conformation illustrated in Fig. 2. Corresponding β-D-mannose 1-phosphate derivatives would have a *cis* arrangement of groups at the 1- and 2-positions, and these groups would have an equatorial–axial relationship. This situation would be very similar but not identical with that in uridine diphosphate glucose, and the nucleotides would resemble each other in their lability towards ammonia.

TDP-rhamnose was much less stable towards ammonia than was the mannose nucleotide. Although quantitative information was not obtained, paper-chromatographic evidence suggested that the rhamnose nucleotide was similar to uridine diphosphate glucose in its lability towards dilute ammonia. It follows that TDP-rhamnose is a β-rhamnose derivative, in which substituents at positions 1 and 2 are in the *cis* relationship, the phosphate being equatorial and the hydroxyl at the 2-position being axial (see Fig. 2). The relationship of substituents in this case is identical with that of β-mannosides.

Confirmation of the structure assigned to TDP-mannose was obtained by synthesis. Thymidine 5′-phosphoramidate was prepared from thymidine 5′-phosphate and ammonia in the presence of di-*cyclo*hexylcarbodi-imide (cf. Moffatt & Khorana, 1958; Chambers & Moffatt, 1958). Reaction between the phosphoramidate and the tri-*n*-octylamine salt of α-D-mannose 1-phosphate occurred readily in pyridine, and thymidine diphosphate mannose was isolated from the mixture of products by ion-exchange chromatography. The synthetic and natural nucleotides were indistinguishable on paper and in their behaviour towards hydrolysis.

Thymidine diphosphate sugars are uncommon. Only recently have communications appeared describing a thymidine diphosphate rhamnose in *Lactobacillus acidophilus* R-26 and *Escherichia coli* 15 T⁻ (Okazaki, 1960), and thymidine diphosphate deoxy sugars in other organisms (Strominger & Scott, 1959). Although it is likely that the rhamnose nucleotide from the *Lactobacillus* is identical with that from *Streptomyces griseus*, no direct comparison has been made and the stereochemistry of the 1-position has not been determined for the former. TDP-mannose has not been described before and, in fact, the only nucleotide containing mannose which has been observed hitherto is guanosine diphosphate mannose.

Experiments on the biochemical significance of TDP-mannose and TDP-rhamnose are in progress, but preliminary results indicate that they are related to each other enzymically. It has been shown recently that an enzyme is present in *Streptococcus faecalis* and in *Pseudomonas aeruginosa* which synthesises TDP-glucose from thymidine triphosphate and α-D-glucose 1-phosphate. Other enzymes in these organisms are able to convert the TDP-glucose into TDP-rhamnose by an unidentified route (Kornfeld & Glaser, 1960; Pazur & Shuey, 1960). We find that an enzyme preparation from an acetone powder of *Streptomyces griseus* is able to convert TDP-mannose into TDP-glucose. Synthetic TDP-glucose was converted by this extract into TDP-mannose, but a simultaneous accumulation of TDP-rhamnose was not observed. However, considerable amounts of a new thymine nucleotide containing an unidentified sugar were formed from both TDP-glucose and TDP-mannose. The mechanism whereby such changes occur has not yet been established experimentally, but the overall process is similar to that in the conversion of guanosine diphosphate mannose into guanosine diphosphate L-fucose (Ginsburg, 1960).

A possible mechanism for the conversion of TDP-glucose into TDP-rhamnose is given in Fig. 3. Inversion and reduction at the 6-position could be achieved by dehydration to a glycoseen (III), followed by prototropic rearrangement to a 4-carbonyl intermediate (IV). The subsequent rearrangement of (IV) to the 3-carbonyl derivative (V) proceeds through a normal enediol intermediate. A spontaneous rearrangement of this type occurs during recrystallization of 2-oxoglucosides (Assarsson & Theander, 1958). Finally, reduction of (V) would give TDP-rhamnose. This last stage is analogous to the reduction of a carbonyl intermediate in the enzymic intercon-

version of uridine diphosphate glucose and uridine diphosphate galactose (Maxwell, 1956; Anderson, Landel & Diedrich, 1956; Kowalsky & Koshland, 1956; Kalckar & Maxwell, 1956).

An interesting feature of this scheme is that one dehydration and one reduction effect three stereochemical inversions. TDP-mannose is not an intermediate in this scheme but could be related to TDP-glucose by oxidation and reduction at the 2-position.

Interconversion of the thymidine derivatives would not necessarily involve change at the 1-position. If the stereochemistry at this position remains unchanged in the transformation, then the α-D-mannose or α-D-glucose compounds would yield a β-L-rhamnose derivative. This stereochemical relationship is the one actually found in the natural nucleotides, and retention of stereochemical arrangement at position 1 supports the view that the interconversion of glucose, mannose and rhamnose occurs whilst they are in combination with nucleotides. The interconversion of guanosine diphosphate mannose and guanosine diphosphate fucose could occur along similar

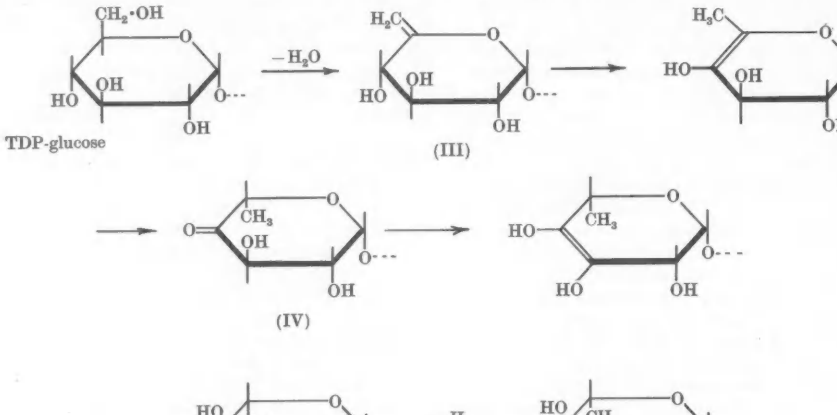


Fig. 3. Suggested mechanism for the conversion of TDP-glucose into TDP-rhamnose.

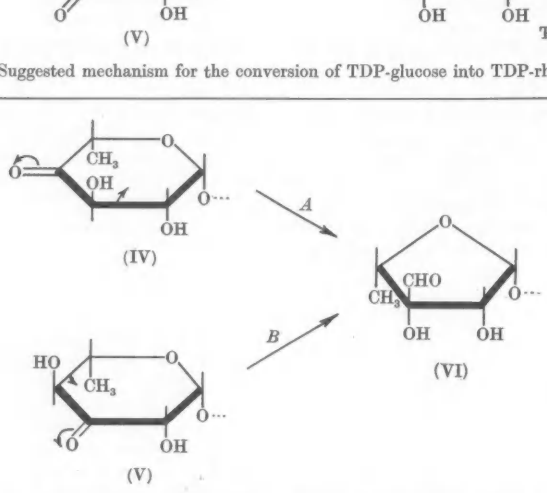


Fig. 4. Possible relationship between rhamnose and streptose derivatives.

lines. We are grateful to Dr J. G. Buchanan for valuable suggestions and discussion of this scheme.

Neither Kornfeld & Glaser nor Pazur & Shuey demonstrated the natural occurrence of TDP-glucose in extracts of the organisms which they studied. However, one of the fractions obtained by us from the ion-exchange chromatography of the nucleotides from *Streptomyces griseus* is a mixture of thymidine and uridine derivatives, which on acid hydrolysis yields glucose and probably fructose. Although this mixture has not yet been resolved it is likely that one of its components is TDP-glucose.

It is possible that both TDP-mannose and TDP-rhamnose are required in *Streptomyces griseus* for the synthesis of polysaccharides, since both mannose and rhamnose are present in the polysaccharide fraction from this organism (Kwapinski & Merkel, 1957). It is also possible that TDP-mannose would be concerned in the introduction of the D-mannosyl residue into streptomycin B (α-D-mannopyranosyl-streptomycin) (Fried & Titus, 1948; Staveley & Fried, 1949).

Another possible function of TDP-rhamnose or its precursors is in the biosynthesis of streptose, the unstable branched-chain sugar component of streptomycin. In Fig. 4 a simple rearrangement is given whereby a thymidine diphosphate streptose might be formed from TDP-rhamnose intermediates.

The carbonyl precursors (IV) and (V) could rearrange as shown to give the branched-chain sugar (VI), which correctly represents streptose (Fried, Walz & Wintersteiner, 1946; Kuehl, Clark, Bishop, Flynn & Folkers, 1949). In route *A* fission of the bond between $C_{(3)}$ and $C_{(3)}$ occurs, a new bond being formed between $C_{(2)}$ and $C_{(4)}$. In route *B* fission occurs between $C_{(4)}$ and $C_{(5)}$, the new bond being between $C_{(3)}$ and $C_{(5)}$. This rearrangement has some similarity to the pinacol–pinacolone transformation and to the glycol rearrangement postulated by Woodward (1957) for the biogenesis of a *C*-formyl group in the antibiotic magnamycin.

The biosynthetic scheme requires the existence of a thymidine diphosphate streptose. No compound of this structure has yet been characterized amongst the complex mixture of nucleotides in *Streptomyces griseus*, but it is known that unidentified thymidine compounds are present in this mixture. Moreover, a streptose nucleotide in which the streptose residue is in β-linkage with the pyrophosphate group would be particularly unstable, since the pyrophosphate in (VI) is in very close proximity to the *cis* hydroxyl at the 2-position. A comparable stereochemical arrangement at positions 1 and 2 occurs in ribose 5-phosphate 1-pyrophosphate.

SUMMARY

1. The nucleotide mixture obtained by alcoholic extraction of *Streptomyces griseus* contains several thymidine derivatives.

2. Thymidine diphosphate mannose and thymidine diphosphate rhamnose have been isolated from this source and their structures have been established. Products detected in their acid hydrolysates include thymine, inorganic phosphate and mannose or rhamnose. The two hexoses were identified chromatographically, and by reduction to the corresponding hexitols followed by treatment with acid. Hydrolysis with rattlesnake venom gave thymidine.

3. The mannose possesses the D-configuration, since the nucleotide, after oxidation with periodate then bromine water followed by acid hydrolysis, gave D-glyceric acid. The configuration of the glyceric acid was established by enzymic phosphorylation and determination of D-glyceric acid 3-phosphate.

4. The above method for determining the configuration of sugar phosphate residues is generally applicable.

5. The stability of the mannose nucleotide towards ammonia, and the instability of the rhamnose nucleotide under comparable conditions, indicate that the former is an α-mannose derivative and the latter is a β-rhamnose derivative. Conformational aspects of these nucleotide structures are discussed.

6. A chemical synthesis of thymidine diphosphate mannose is described.

7. The enzymic conversion of thymidine diphosphate glucose into thymidine diphosphate mannose has been observed and a possible mechanism for the biosynthesis of rhamnose is given.

8. Other likely biochemical functions for the thymidine derivatives are discussed. These include a suggested scheme for the biosynthesis of streptose.

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The Ribitol Teichoic Acid from *Lactobacillus arabinosus* Walls: Isolation and Structure of Ribitol Glucosides

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It has been known for some time that considerable amounts of a ribitol teichoic acid occur in the walls of *Lactobacillus arabinosus* 17–5 (Baddiley, Buchanan & Greenberg, 1957; Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan & Carss, 1958*a*; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958*b*). Preliminary studies, carried out largely with the aid of paper chromatography, suggested that this compound is a polymer in which ribitol units are joined together through phosphodiester linkages, and that glucosyl and alanine ester groups are also present (Armstrong *et al.* 1958*b*). It is clear that this teichoic acid resembles the ribitol teichoic acid in the walls of *Bacillus subtilis* (Armstrong, Baddiley & Buchanan, 1960, 1961), but several differences in the detailed structure of the two polymers were observed in the preliminary work. Hydrolysis with acid and alkali

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revealed that, whereas the compound from *B. subtilis* is a regular polymer in which 4-*O*-(β-D-glucopyranosyl)-D-ribitol units are joined through phosphodiester groups, that from *L. arabinosus* contains mono- and di-glucosylribitol units. Preparations from some batches of micro-organisms also contain ribitol units which bear no sugar. Moreover, whereas the glycosidic linkages in the teichoic acid from *B. subtilis* have the β-configuration, those in the *L. arabinosus* polymer probably have the α-configuration.

A more detailed investigation has now been carried out on the ribitol teichoic acid from *L. arabinosus*, and the earlier conclusions have been substantiated. In this paper the isolation and structure of ribitol mono- and di-glucosides from hydrolysates of the teichoic acid is described.

EXPERIMENTAL

Materials. β-Glucosidase was kindly provided by Dr W. J. Whelan; crude yeast hexokinase was a gift from Sigma Chemical Co., St Louis, Mo., U.S.A.; calf intestinal phosphomonoesterase was purchased from Sigma Chemical Co.; DL-glyceric acid, adenosine triphosphate and reduced diphosphopyridine nucleotide were purchased from L. Light and Co. Ltd., Colnbrook, Bucks.; Glucostat reagent was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; prostatic phosphatase was prepared from human prostate glands; *L. arabinosus* 17–5 was grown from a culture supplied by Dr D. E. Hughes.

Methods

Paper chromatography. Paper chromatography was carried out on Whatman no. 1, 4, and 3 mM paper that had been washed with 2N-acetic acid and then water. The following solvent systems were used: *A*, propan-1-ol–ammonia (sp.gr. 0·88)–water (6:3:1) (Hanes & Isherwood, 1949); *B*, butan-1-ol–ethanol–water–ammonia (sp.gr. 0·88) (40:10:49:1) (organic phase) (Hirst, Hough & Jones, 1949); *C*, ethyl acetate–pyridine–water (10:4:3) (Jermyn & Isherwood, 1949).

The products were detected by the periodate–Schiff reagents for α-glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949), aniline phthalate reagent for reducing sugars (Partridge, 1949), silver nitrate reagent for reducing sugars (Trevelyan, Proctor & Harrison, 1950) and ninhydrin reagent for amino acids (Consden & Gordon, 1948).

Paper electrophoresis. Paper electrophoresis was carried out on washed Whatman no. 4 paper in 0·05M-sodium tetraborate at 5v/cm. for 16 hr. The glucosides were located by spraying with 10% acetic acid in butanol, allowing to dry and then spraying with 1% sodium metaperiodate solution containing 5% of acetic acid and developing with the Schiff reagent in the usual manner.

Isolation of teichoic acid from Lactobacillus arabinosus *cells. L. arabinosus* 17–5 was grown in 9 l. batches for 16 hr. at 30° in the following medium: Oxoid Tryptone, 20 g.; Oxoid yeast extract, 10 g.; sodium acetate, 10 g.; glucose, 20 g.; potassium dihydrogen phosphate, 4·5 g.; sodium hydroxide, 1·04 g.; inorganic salts (Barton-Wright, 1946), 5 ml.; distilled water, 100 ml.

The cells (about 80 g. wet wt.) were harvested in a refrigerated Sharples centrifuge and washed with 0·95% sodium chloride solution at 0°. They were treated (30 sec.) in a blender with successive portions of acetone, ethanol and ether (15 vol. of each) at room temperature.

Fat-free cells (160 g.) were extracted by blending for 1 min. with 10% trichloroacetic acid (600 ml.) at 2°. The residue, after removal of the supernatant solution by centrifuging, was extracted twice in a similar manner. The combined extracts were filtered through Celite. To the clear yellow solution (1·5 l.) cold ethanol (2 l.) was added with stirring and the mixture stored at 0° for 24 hr., when the resultant yellow–white precipitate was removed by centrifuging and redissolved in cold 10% trichloroacetic acid (120 ml.). After removal of insoluble material, cold ethanol (150 ml.) was added and, after 24 hr. at 0°, the precipitated teichoic acid was removed by centrifuging and dried with ethanol and ether. Yield, 667 mg. of powder composed largely of ribitol teichoic acid, together with some glycerol teichoic acid and nucleic acid.

The residues from the above extraction were resuspended in 10% trichloroacetic acid (700 ml.) and blended for 1 min. at 0°. The suspension was then stirred at 0° for 24 hr. and again blended for 1 min. before centrifuging. The resulting residue was stirred with 10% trichloroacetic acid (300 ml.) and again centrifuged, the combined solutions then being filtered through Celite and mixed with 1·5 vol. of cold ethanol. After 24 hr. at 0° the precipitated material was collected as before. At this stage the material contained a large amount of nucleic acid, much of which was removed by extraction of the teichoic acid with ice-cold distilled water (100 ml.). The insoluble material (416 mg.) was removed by centrifuging and teichoic acid (1·316 g.) was obtained by precipitation with ethanol. This material contained ribitol teichoic acid and a little glycerol teichoic acid and nucleic acid. The debris, which still contained teichoic acid, was not extracted further.

Isolation of teichoic acid from Lactobacillus arabinosus *walls.* The cells were grown for 16 hr. as described above and, after washing with cold 0·95% sodium chloride, were suspended in cold distilled water (20 g. wet wt. in 100 ml. of water). Walls were prepared as described by Armstrong *et al.* (1960). The yield from a 9 l. culture was 0·8–1·4 g. (Found: P, 2·87–3·3%). No whole cells were detected on examination by electron microscopy. Samples (3 mg.) were hydrolysed for 3 hr. at 100° with 2N-hydrochloric acid. After removal of hydrochloric acid by evaporation over potassium hydroxide, the residues were chromatographed in solvent *A*. The walls (204 mg.) were stirred for 24 hr. in 10% trichloroacetic acid at 2°. After centrifuging the wall residues were suspended in trichloroacetic acid and re-extracted. Ethanol (2 vol.) was added to the combined solutions and the resulting precipitate (42 mg.) collected as before. This material was pure ribitol teichoic acid. Acetone (2 vol.) was added to the supernatant from this precipitation; the resulting precipitate (23 mg.) contained teichoic acid and polysaccharide. The wall residues (125 mg.) were freed from trichloroacetic acid by extraction with ether.

In a similar extraction of walls (412 mg. Found: P, 2·87%), 94 mg. of pure teichoic acid (Found: P, 6·5%),

22·4 mg. of teichoic acid–polysaccharide mixture (Found: P, 5·8%) and 200 mg. of extracted wall residue (Found: P, 0·7%) were obtained. In addition, the supernatant from the acetone precipitation contained 2·4 mg. of phosphorus, which corresponded to partially degraded or low-molecular-weight teichoic acid.

Examination of extracted walls. Extracted walls (3·7 mg.) were hydrolysed in 2N-hydrochloric acid (300 μl.) for 24 hr. at 100°. After evaporation to dryness, the hydrolysate was passed through columns (0·5 ml.) of Dowex 50 (H^+ form) and Dowex 2 (CO_3^{2-} form) resins. The eluate was evaporated to dryness and examined chromatographically (solvent *A*), together with unextracted walls (1·3 mg.) which had been treated similarly. The amount (visual estimation) of anhydroribitol present in the hydrolysate of the extracted walls was less than that present in the unextracted walls containing an equivalent amount of phosphorus.

Extracted walls (46 mg.) were re-extracted by stirring with two successive portions (20 ml.) of 10% trichloroacetic acid at 70°. The wall residue (40 mg.), after removal of trichloroacetic acid by washing with ether, contained 0·36% of phosphorus. No anhydroribitol was detected when this residue (8·9 mg.) was examined as above. Electron microscopy showed that the walls had not disintegrated appreciably. Further extraction at 90° for 1 hr. with three successive portions of 10% trichloroacetic acid left a residue containing 0·04% of phosphorus. After this treatment the walls had disintegrated completely to a granular material. It appears that *L. arabinosus* walls contain small amounts of phosphorus other than that constituting teichoic acid. The nature of this phosphorus is not known.

Teichoic acid

Acid hydrolysis. Teichoic acid (2·0 mg.) isolated from walls was hydrolysed in 2N-hydrochloric acid in a sealed tube for 3 hr. at 100°. After evaporation the samples were examined by paper chromatography in solvents *A*, *B*, and *C*. Products are given in Table 1.

Analysis. Teichoic acid (1·5 mg.), isolated from walls, was hydrolysed in 2N-hydrochloric acid (200 μl.) for 4 hr. at 100°. The hydrolysate was neutralized with ammonia and freeze-dried, and the residue was dissolved in water (5·0 ml.). Phosphorus (Chen, Toribara & Warner, 1956), glucose (Park & Johnson, 1949) and alanine (Rosen, 1957) were determined. The glucose value was corrected for destruction (5%) by acid during hydrolysis (Found: P, 6·44; glucose, 40·3; alanine, 12·1%. Ratio P:glucose: alanine, 1:1·06:0·62). The reducing sugar present in an acid hydrolysate of teichoic acid was shown by assay with the Glucostat reagent to be 98% of D-glucose.

Table 1. *Products obtained by hydrolysis of teichoic acid*

Teichoic acid was hydrolysed for 3 hr. in 2N-HCl or N-NaOH at 100°. The products were identified by chromatography.

	Acid hydrolysis	Alkali hydrolysis
1:4-Anhydroribitol	+	+*
Ribitol	+	+*
Glucose	+	−
Alanine	+	+
Glucosylribitol	−	+*
Diglucosylribitol	−	+*
Ribitol phosphates	+	+*
Glucosylribitol phosphates	−	+
1:4-Anhydroribitol 5-phosphate	+	−
Inorganic phosphate	+	−

* Trace.

Action of phosphodiesterase on teichoic acid. Teichoic acid (1·7 mg.), prepared from cell walls, was dissolved in water (3·0 ml.) and mixed with freeze-dried *Crotalus atrox* venom (0·4 mg.) dissolved in 0·1M-glycine buffer, pH 9·0 (1 ml.), and 1 mM-magnesium sulphate (0·3 ml.). The mixture was then incubated at 37° for 24 hr. before being passed through a small column (0·5 ml.) of Dowex 50 (H^+ form) resin and then adjusted to pH 5·5 with aqueous ammonia solution. The solution was freeze-dried, 0·2M-sodium acetate buffer, pH 5·5 (2 ml.), containing prostatic phosphatase (0·1 mg.), was added, and the volume was adjusted to 5 ml. with water. After incubation for 24 hr. at 37°, the ratio inorganic: total phosphate was 0·145:1. After similar incubation with *Crotalus adamanteus* venom, the ratio was 0·093:1. A sample of teichoic acid which was incubated with prostatic phosphatase without previous treatment with snake venom gave after 48 hr. a ratio of 0·10:1. On incubation with a purified calf intestinal phosphomonoesterase, the ratio was 0·145:1.

Alkali hydrolysis and enzymic dephosphorylation. Teichoic acid (980 mg.), isolated from whole cells by extraction at 2°, was dissolved in N-sodium hydroxide solution (20 ml.) and the resulting solution was heated at 100° for 3 hr. A red–brown precipitate (probably ferric hydroxide) was removed by centrifuging. The combined supernatant and washings (50 ml.) were passed through a Dowex 50 (NH_4^+) resin column (30 ml.), which was then washed with water (200 ml.). The eluate and washings were concentrated to 10 ml. *in vacuo* or, in a second experiment, by freeze-drying. A sample (0·1 ml.) was removed and examined chromatographically in solvents *A* and *B* (see Table 1).

The pH of the eluate was adjusted to 5·5 with acetic acid, 0·2M-sodium acetate buffer, pH 5·5 (10 ml.), was added, and the solution was adjusted to 50 ml. with water; 4% of the phosphorus present was inorganic. Prostatic phosphatase (1·5 ml.) was added and the mixture was kept at 37°. Samples were removed for analysis after 24 hr. (inorganic P, 69% of total P) and 48 hr. (inorganic P, 70% of total P). A further portion of phosphatase (1·0 ml.) was added and incubation was continued for 24 hr. (inorganic P, 69% of total P). The volume was adjusted to 200 ml. with water and the solution was passed through a column of Dowex 50 (H^+ form) resin (70 ml.) at 2°. The filtrate, together with 250 ml. of column washings, was shaken with Dowex 2 (CO_3^{2-} form) resin (20 ml.), and added to a column of Dowex 2 (CO_3^{2-}) resin (20 ml.), which was then washed with water (400 ml.). The eluate was evaporated under reduced pressure to small volume and freeze-dried. The resulting white solid (288 mg.) was examined chromatographically in solvents *A*, *B* and *C*. Phosphates (520 mg.) were eluted from the Dowex-2 resin with N-sodium hydroxide solution (125 ml.) at 2°. They were isolated by shaking the eluate at 2° with Dowex 50 (H^+) (100 ml.), adding both the supernatant and resin to a column of Dowex 50 (H^+) resin (150 ml.) at 2°, washing with water (1 l.), neutralizing with ammonia and freeze-drying. Those compounds listed in Tables 2 and 3 were detected.

Examination of degradation products

Fractionation of the neutral products. (*a*) On a cellulose column. The neutral fraction (280 mg.) was dissolved in solvent *B* (50 ml.) and applied to a Chromax paper column which had previously been washed with solvent. After washing with three successive portions (10 ml.) of solvent, elution was commenced. Fractions (15 ml.) were collected, and the elution of products was followed qualitatively by paper chromatography and quantitatively by micro-periodate oxidation (Dixon & Lipkin, 1954) on 0·1 ml. samples of eluate after removal of solvent *in vacuo*.

Most of the fractions contained mixtures, the composition of which was determined by descending chromatography on Whatman 3mM paper with solvent *B* (see Table 2).

(*b*) On a charcoal–Celite column. A sample of teichoic acid (500 mg.) was treated with alkali and prostatic phosphomonoesterase as before, and the neutral fraction from this was dissolved in water and passed through a column of equal parts of charcoal and Celite (total weight, 50 g.). The elution of products was followed by paper chromatography, and appropriate fractions were combined and evaporated under reduced pressure (Table 3). Mixtures were separated on Whatman 3mM paper. The 3 glucoside was obtained pure only after repeated chromatographic separation from the 4-glucoside. After this purification all sugar derivatives were adsorbed on short charcoal–Celite columns and eluted with 15% ethanol. The solvent was removed under reduced pressure and the product finally freeze-dried.

Unhydrolysed phosphates. The unhydrolysed phosphates consisted mainly of a monophosphate of diglucosylribitol.

Table 2. *Cellulose separation of products from the action of alkali and phosphatase on teichoic acid*

For details see text.

Fraction no.	Composition
1–59	—
60–97	Anhydroribitol, glycerol
98–145*	Ribitol, 4-glucosylribitol, glucose (trace)
146–155	4-Glucosylribitol
156–226	4-Glucosylribitol, 3-glucosylribitol
227–300†	Mono- and di-glucosylribitol
301–460	Diglucosylribitol

* Also contained a trace of a compound with $R_{ribitol}$ 1·19 (solvent *B*).

† Also contained a trace of a compound with $R_{ribitol}$ 0·29 (solvent *B*).

1:4-*Anhydroribitol.* The eluate from the charcoal column (tubes 151–176) was evaporated to dryness *in vacuo* and the anhydroribitol was purified by sublimation at 145° and 10^{-4} mm. The resulting syrup crystallized after the addition of a seed crystal of DL-anhydroribitol. Yield, about 1·5 mg. of white crystals. The infrared spectrum was identical with that of authentic D-1:4-anhydroribitol. Polarimetric examination in a 1 dm. tube of a solution containing about 0·5 mg. in 1·4 ml. of water gave a rotation of +0·02–0·03°.

Ribitol. Chromatographically pure ribitol crystallized from ethanol as rosettes of white needles (23 mg.), m.p. 99° (Found: C, 39·8; H, 8·2. Calc. for $C_5H_{12}O_5$: C, 39·5; H, 7·9%). The infrared spectrum was identical with that of an authentic sample of ribitol.

Identification of 4-*O*-(α-D-*glucopyranosyl*)-D-*ribitol*

Chromatographically pure 4-glucosylribitol crystallized with difficulty from aqueous ethanol as rosettes of white needles (22 mg.), m.p. 125–126°, $[\alpha]_D^{22}$ +106° in water (*c*, 1·2) (Found: C, 40·0; H, 7·8. $C_{11}H_{22}O_{10},H_2O$ requires C, 39·8; H, 7·2%. Ratio ribitol:glucose, found 1:0·94). It showed a band at 1631 $cm.^{-1}$ in the infrared, characteristic of compounds containing water of crystallization.

Acid hydrolysis. Samples of the glucoside (about 0·5 mg.) were hydrolysed in 2N-hydrochloric acid (100 μl.) for 3 hr. and 24 hr. The hydrolysates were neutralized with ammonia and examined by paper chromatography in solvent *A*. After 3 hr. the glucoside was completely hydrolysed to ribitol, anhydroribitol and glucose; after 24 hr. the products were anhydroribitol and glucose.

Periodate oxidation and borohydride reduction. The glucosylribitol (1·0 mg.) was dissolved in 1% sodium metaperiodate solution (0·4 ml.) and the solution kept at room temperature for 24 hr. Sodium borohydride (2·0 mg.) was then added; after a further 24 hr. 2N-hydrochloric acid (0·2 ml.) was added and the solution was heated at 100° for 30 min. After evaporation under reduced pressure the mixture was examined by ascending chromatography in ethyl acetate–pyridine–water (7:2:1) (Viscontini, Hoch & Karrer, 1955). A single product was observed which rapidly gave a purple colour with the periodate–Schiff reagents and had R_F 0·45 (glycerol had R_F 0·45, erythritol had R_F 0·37 and ethylene glycol had R_F 0·58).

Quantitative periodate oxidation. Glucosylribitol (7·4 mg.) was dissolved in water (10 ml.), 0·04M-sodium metaperiodate (5 ml.) was added, and the volume adjusted to 20 ml. with water. A control experiment containing no glucosylribitol was also performed. Solutions were kept in the

Table 3. *Charcoal chromatography of products from the action of alkali and phosphatase on teichoic acid*

For details see text.

Fraction no.	Eluent	Composition
1–14	Water	—
15–29		Glycerol
30–39		Glycerol, ribitol
40–83		Ribitol
84–130		—
131–150	2% Ethanol	Anhydroribitol (trace)
151–176		Anhydroribitol
177–200		Anhydroribitol (trace)
201–221	3% Ethanol	3- and 4-Glucosylribitol
222–247	4% Ethanol	4-Glucosylribitol
248–320	6% Ethanol	3- and 4-Glucosylribitol, diglucosylribitol
321–390	7·5% Ethanol	Diglucosylribitol
391–411	9·0% Ethanol	Diglucosylribitol (trace)

dark at room temperature and samples were withdrawn at intervals for determination of periodate (4 ml. samples), formic acid (5 ml. samples) and formaldehyde (0·4 ml. samples) (Hough, Powell & Woods, 1956). The formic acid was titrated with 0·01 N-sodium hydroxide solution to pH 6·4. After 24 hr. the glucosylribitol had consumed 4·00 mol.prop. of periodate and 1·02 mol.prop. of formaldehyde had been produced. After 48 hr. 4·10 mol.prop. of periodate had been consumed and 1·06 mol.prop. of formaldehyde and 1·43 mol.prop. of formic acid were produced. On addition of an excess of sodium hydroxide solution and back-titration with 0·01 N-oxalic acid, the value obtained for formic acid was 1·90 mol.prop.

Oxidation and hydrolysis to glyceric acid. The glucosylribitol (1·6 mg.) and α-methyl glucoside (2·0 mg.) were separately dissolved in 0·15 M-sodium metaperiodate (0·5 ml.), and the solutions were kept at room temperature for 48 hr. to ensure complete oxidation. The solutions were passed through columns of Dowex 50 (H^+ form) resin (0·5 ml.) and Dowex 2 (acetate form) resin (0·5 ml.). The columns were washed with water (10 ml.), the eluates were freeze-dried, and acetic acid was removed *in vacuo*. The residues were dissolved in a solution (2 ml.), 100 ml. of which contained 417 mg. of benzoic acid and 565 mg. of barium hydroxide. Bromine (0·02 ml.) was added to the ice-cold solutions which were then shaken in stoppered tubes for 1 hr. and kept for 60 hr. at room temperature. Air was passed through the solutions to remove excess of bromine and the volumes were reduced to 0·8–1·0 ml. by evaporation under reduced pressure. Benzoic acid was removed by centrifuging and washed with a little ice-cold water. The volume of combined filtrate and washings was reduced *in vacuo* to 0·5 ml., 5 N-sulphuric acid (0·07 ml.) was added to each tube, and the solutions were heated at 100° for 2 hr. Barium carbonate (40 mg.) was added to each and the mixtures were shaken at intervals during 24 hr. Insoluble barium salts were removed by centrifuging and washed with water. The supernatant and washings were passed through columns of Dowex 50 (NH_4^+ form) resin (0·5 ml.) and evaporated to dryness *in vacuo*. The glyceric acid was purified by chromatography in solvents *A* and *B*. Paper strips containing glyceric acid, together with corresponding control areas of paper, were cut out and eluted with water or 0·01 N-ammonia solution.

The eluates were diluted to 2·0 ml. with water. Samples were removed for chemical assay of the glyceric acid by chromotropic acid (Bartlett, 1959) or by oxidation with 0·1 mM-sodium metaperiodate (Dixon & Lipkin, 1954). Oxidation was complete in 1 hr., 1 mole of periodate being consumed by each mole of glyceric acid. Samples (0·2–0·4 ml.) were then mixed with 3·5 ml. of a solution composed of 0·01 M-phosphate buffer, pH 7·3 (12·0 ml.), reduced diphosphopyridine nucleotide (about 4·0 mg.), 0·1 M-magnesium sulphate (0·2 ml.), 0·2 M-adenosine triphosphate (0·2 ml.), rabbit-muscle enzyme (10 mg.) and yeast hexokinase (12 mg.). In a control experiment, water (0·2–0·4 ml.) was mixed with 3·5 ml. of the enzyme solution.

Oxidation of the pyridine nucleotide was followed spectrophotometrically at 340 mμ, and under the above conditions was usually complete after 30 min. Oxidation was calculated from the change in extinction at 340 mμ.

The glyceric acid from α-methyl glucoside oxidized 2·0 mol.prop. of pyridine nucleotide, whereas that from the glucosylribitol oxidized 1·9 mol.prop. Authentic D-glyceric acid, prepared by enzymic dephosphorylation of D-glyceric acid 3-phosphate, oxidized 2 mol.prop. of pyridine nucleotide, whereas DL-glyceric acid oxidized 1 mol.-prop. of nucleotide at a reduced rate.

Identification of 3-*O*-(α-D-*glucopyranosyl*)*ribitol*

Chromatographically purified 3-*O*-(α-D-glucopyranosyl)-ribitol, from which all 4-glucosylribitol had been removed, was adsorbed on a charcoal–Celite column (300 mg.) which was then washed with water, and the glycoside was eluted with 10% ethanol. The eluate was evaporated to dryness under reduced pressure and the residue was dissolved in water, then freeze-dried to a white powder (1·7 mg.). It had $[\alpha]_D^{20} + 78 \pm 20°$ in water (*c*, 0·284) and a ratio glucose: ribitol of 0·97:1. The amount of glucosylribitol in this solution, as estimated by determination of glucose in an acid hydrolysate, was 1·55 mg.

The solution was quantitatively transferred to a test tube and the volume adjusted to 3·25 ml. with water; the concentration of glucosylribitol in this solution, determined by estimation of the glucose produced on acid hydrolysis, was 0·421 mg./ml. The remainder of the solution was used for quantitative periodate oxidation studies.

Controlled oxidation with sodium metaperiodate. The glucosylribitol solution (1·0 ml.) was mixed with 6 mM-sodium metaperiodate solution (2 ml.) and kept in the dark at room temperature. A control containing no glucosylribitol was also prepared. Samples (0·20 ml.) of each solution were diluted to 10 ml. with water and the periodate content was determined at intervals. Formaldehyde was determined in other samples (0·4 ml.). After 6 hr., 4·1 mol.prop. of periodate were consumed, whereas after 24 hr. 4·8 mol.prop. of periodate were consumed and 2·2 mol.prop. of formaldehyde were formed. After 72 hr., 5·1 mol.prop. of periodate were consumed and 2·3 mol.prop. of formaldehyde were formed.

Oxidation with sodium metaperiodate at pH 8·0. The glucosylribitol solution (0·35 ml.) was mixed with 0·1 M-phosphate buffer, pH 8·0 (1·70 ml.), 0·3 M-sodium metaperiodate (0·27 ml.) and water (1·00 ml.). A control experiment containing no glucosylribitol was also prepared and both solutions were kept in the dark at 37°. Samples (0·4 ml.) were removed for determination of formaldehyde. After 12 hr., 3·12 mol.prop. and after 24 hr. 3·03 mol.prop. of formaldehyde were produced.

Identification of 3:4-*di*-(O-α-D-*glucopyranosyl*)-D-*ribitol*

Diglucosylribitol was precipitated by cooling its solution in aqueous ethanol. It was a deliquescent, microcrystalline, white powder (30 mg.) which changed form at 106° and melted with decomposition at 118–121°. It had $[\alpha]_D^{22} + 137°$ in water (*c*, 1·08) (Found: C, 42·2; H, 7·1. $C_{17}H_{32}O_{16}$,$6H_2O$ requires C, 41·6; H, 6·8%. Glucose:ribitol ratio, found 1·92:1). The hygroscopic nature of the material prevented satisfactory measurement of its infrared spectrum, but a strong absorption at 1639 cm.$^{-1}$, characteristic of hydrated compounds, was observed.

Acid hydrolysis. Diglucosylribitol (about 0·5 mg.) was hydrolysed in 0·1 N-hydrochloric acid (100 μl.) for 90 min. The hydrolysate was neutralized with aqueous ammonia solution and examined chromatographically in solvent *B*.

Unhydrolysed diglucosylribitol (about 50%), glucose, and approximately equal parts of 3- and 4-glycosylribitol were the only compounds detected. In 2N-hydrochloric acid at 100° for 4 hr. the products were glucose, ribitol and anhydroribitol.

Periodate oxidation and borohydride reduction. Acid hydrolysis of the reduced product of periodate oxidation gave two glycols; the principal one had R_F 0·45 and the minor one had R_F 0·39 (glycerol had R_F 0·45 and erythritol had R_F 0·37).

Quantitative periodate oxidation. Diglucosylribitol (4·99 mg.) was dissolved in a little water, 0·02M-sodium metaperiodate solution (5 ml.) was added and the volume was adjusted to 25 ml. with water. The solution, and a control solution, were kept in the dark at room temperature and samples were removed for analysis. After 36 hr., 5·10 mol.prop. of periodate were consumed and 1·80 mol.prop. of formic acid were formed; after 72 hr., 5·21 mol.prop. of periodate were consumed and 1·98 mol.prop. of formic acid and 1·08 mol.prop. of formaldehyde were formed. The oxidation product was stable at pH 6·9, but was unstable above pH 7, giving rise to acidic products.

Partial acid hydrolysis. The diglucoside (10 mg.) was dissolved in 0·1N-hydrochloric acid and heated at 100° for 90 min. The solution was neutralized with ammonia solution and ions were removed by use of a charcoal–Celite column. The resulting monoglucosylribitols were purified by chromatography in solvent *B*, adsorption on charcoal and elution with ethanol as before, and identified as follows:

(*a*) 4-*O*-(α-D-Glucopyranosyl)-D-ribitol. This compound, obtained by hydrolysis of the diglucoside, was indistinguishable on paper chromatography (solvent *B*) and colour reactions from authentic 4-glucosylribitol. On acid hydrolysis, glucose, ribitol and anhydroribitol were the only products detected. The glucose:ribitol ratio was 0·92:1. The pure glucosylribitol (0·8 mg.) was oxidized with periodate, the product was reduced with borohydride then hydrolysed, and fragments were examined as before. Glycerol (R_F 0·45) was the only product detected with the periodate–Schiff reagents. A sample (1·2 mg.) of the glucosylribitol was oxidized with sodium metaperiodate followed by bromine, then hydrolysed and the resulting glyceric acid was examined as before. With α-methyl glucoside as a reference compound, the enzymic procedure showed that 90% of the glyceric acid formed had the D-configuration.

(*b*) 3-*O*-(α-D-Glucopyranosyl)ribitol. This compound from the partial acid hydrolysis gave no reaction with aniline phthalate and had the same chromatographic properties in solvent *B* as had 3-*O*-(α-D-glucopyranosyl)-ribitol. It also gave the characteristic yellow colour with the periodate–Schiff reagents. On acid hydrolysis, glucose, ribitol and anhydroribitol were the only products detected. The glucose:ribitol ratio was 0·97:1. On oxidation with sodium metaperiodate at pH 8·0, 2·80 mol.prop. of formaldehyde were produced.

DISCUSSION

Analysis of this teichoic acid and paper-chromatographic characterization of hydrolysis products were carried out on material which had been prepared from isolated walls as well as from whole cells. The walls were obtained by mechanical rupture of bacteria with a Mickle vibrator followed by differential centrifuging, the technique being adapted from that of Salton & Horne (1951). This method was the most satisfactory one for preparing cell walls from *L. arabinosus* for the present purpose. Much of the teichoic acid could be extracted from walls by treatment with cold 10% trichloroacetic acid at 2° for several hours. Extraction at 37°, or even higher temperatures, was more complete but these vigorous conditions led to the presence of contaminants in the final product. The ease of extraction of this teichoic acid is comparable with that for *Bacillus subtilis* (Armstrong *et al.* 1960), and is consistent with the view that most, or possibly all, of the teichoic acid in bacterial walls is held in the wall structure by ionic linkages.

L. arabinosus walls contain about 3% of phosphorus, 90% of which can be accounted for as ribitol teichoic acid after extraction with trichloroacetic acid solution. The nature of the small amount of unextracted phosphorus is unknown. This organic phosphate is not a teichoic acid. Similar amounts of unextracted organic phosphates have been observed in the walls of other bacteria studied in this laboratory.

Pure teichoic acid was obtained from the extract by precipitation with ethanol, dissolving in water and reprecipitation with ethanol. It contained 6·44% of phosphorus and gave on acid hydrolysis alanine, glucose, inorganic phosphate and the known acid-degradation products of ribitol phosphate (Armstrong *et al.* 1958*b*). The ratio phosphorus:glucose:alanine was 1:1·06:0·62. The amount of alanine depended upon the conditions of preparation but the molar proportion was always less than 1. At present it is assumed that a molar proportion of about 1 occurs in teichoic acid within the wall and that partial loss of alanine takes place during isolation.

A sample of alanine from this teichoic acid was readily oxidized by kidney D-amino acid oxidase (experiment by Dr F. C. Neuhaus) and thus has the D-configuration, a feature common to all teichoic acids examined in this Laboratory. Moreover, the alanine residues react readily at room temperature with dilute ammonia or hydroxylamine, giving alanine amide and hydroxamate respectively. These observations, and the presence of a typical ester carbonyl absorption band at 1751 cm.$^{-1}$ in the infrared, indicate that the alanine residues are joined through their carboxyl groups to hydroxyl groups of either the glucose or ribitol residues. It has been shown that the alanine residues in the ribitol teichoic acid from *B. subtilis* are joined to ribitol and not to glucose (Armstrong *et al.* 1961).

The general properties of this polymer and the nature of the products of its acid hydrolysis are

consistent with a structure in which ribitol residues are joined together through phosphodiester linkages. This similarity to the ribitol teichoic acid from *B. subtilis* walls is in contrast with the nature and number of glucosyl residues attached to ribitol. When the phosphodiester linkages in the *B. subtilis* polymer were hydrolysed by alkali, a mixture of phosphomonesters was produced almost quantitatively. Removal of phosphate from the esters through the action of prostatic phosphatase gave a high yield of 4-*O*-(β-D-glucopyranosyl)-D-ribitol, and it was concluded that the teichoic acid is a polymer in which each glucose residue is attached in a regular manner to each ribitol in the chain. When a similar procedure was applied to the teichoic acid from *L. arabinosus*, a mixture of products was obtained. In order to obtain sufficient of these for adequate characterization, larger-scale experiments were carried out on hydrolysates of teichoic acid prepared from extracts of whole, defatted bacteria. Such preparations were less pure than those from walls, and contained hydrolysis products from the intracellular glycerol teichoic acid that is known to be present in this organism (cf. Baddiley & Davison, 1961).

The main products of hydrolysis were a monoglucosylribitol and a diglucosylribitol, and appreciable amounts of ribitol were also obtained from preparations made from some batches of organisms. In addition, small amounts of 1:4-anhydroribitol and an isomeric monoglucosylribitol were detected in the hydrolysate. The anhydroribitol is probably not a structural unit in the teichoic acid, but could arise during the alkali treatment. A trace of 1:4-anhydroribitol is formed by the prolonged action of hot alkali on ribitol 5-phosphate (unpublished observation by Mr D. A. Applegarth), and it is possible that phosphodiesters in a teichoic acid would yield the anhydro-compound more readily than would the simple phosphomonoester. Occasionally a small amount of an isomeric diglucosylribitol has been detected in hydrolysates, but this is not a regular product.

The hydrolysis products were separated from each other by chromatography on charcoal, followed in some cases by preparative paper chromatography. Anhydroribitol was isolated crystalline by sublimation. It had the same chromatographic mobility in several solvent systems as authentic 1:4-anhydroribitol and its infrared spectrum was identical with that of optically active 1:4-anhydroribitol, which differs from the spectrum of the racemate. Insufficient material was available for accurate measurement of its optical activity, but the solution was slightly dextrorotatory, suggesting that the sample was the D-isomer. Ribitol was isolated crystalline and identified by direct comparison with synthetic material. The predominant isomer of glucosylribitol also crystallized as a monohydrate. It had the same R_F as the glucosylribitol from *B. subtilis* teichoic acid in two solvents and behaved similarly on electrophoresis, but differed slightly in the colour given with the periodate–Schiff reagents. The blue colour given by the β-glucoside from *B. subtilis* closely resembles that from β-methyl glucoside, whereas that shown by the glucoside from *L. arabinosus* possesses a blue–grey component. α-Methyl glucoside gives a marked blue–grey colour in this test.

Acid hydrolysis of the glucosylribitol gave glucose, ribitol and anhydroribitol, whereas under more vigorous conditions equimolar amounts of glucose and anhydroribitol were formed. These were separated from each other by paper chromatography. Anhydroribitol was determined by oxidation with periodate, and glucose by reaction with ferricyanide (Park & Johnson, 1949). The high positive rotation ($[\alpha]_D^{22} + 106°$) of this glucosylribitol suggested that it is an α-glucopyranoside. This is consistent with the observation that it was not hydrolysed with the β-glucosidase preparation which readily hydrolysed the 4-*O*-(β-D-glucopyranosyl)-D-ribitol from *B. subtilis*, and is also consistent with the earlier conclusion that the high rotation of the teichoic acid from *L. arabinosus* is due to the presence of α-glucosyl residues. There is thus a marked difference between the teichoic acid from *L. arabinosus* and that from *B. subtilis* in which all glucose linkages possess the β-configuration.

The position of attachment of glucose to ribitol was established by oxidation with sodium metaperiodate; 4 mol.prop. of periodate were consumed and 2 mol.prop. of formic acid and 1 mol.prop. of formaldehyde were produced. For complete liberation of formic acid it was necessary to add an excess of sodium hydroxide. The 4-*O*-(β-D-glucopyranosyl)-D-ribitol from *B. subtilis* teichoic acid behaved similarly, and this is believed to arise from the intermediate formation of a glucosyl-D-erythrose, which then gives a formyl ester of the final oxidation product. The course of the oxidation has been discussed fully (Armstrong *et al.* 1960). It follows from the analysis that the glucose must be attached to a hydroxyl at position 2 or 4 in the ribitol residue (cf. I and II). This conclusion is supported by the observation that the trialdehyde formed by oxidation of the glucoside with periodate, on reduction with borohydride and acid hydrolysis, gave glycerol but not ethylene glycol.

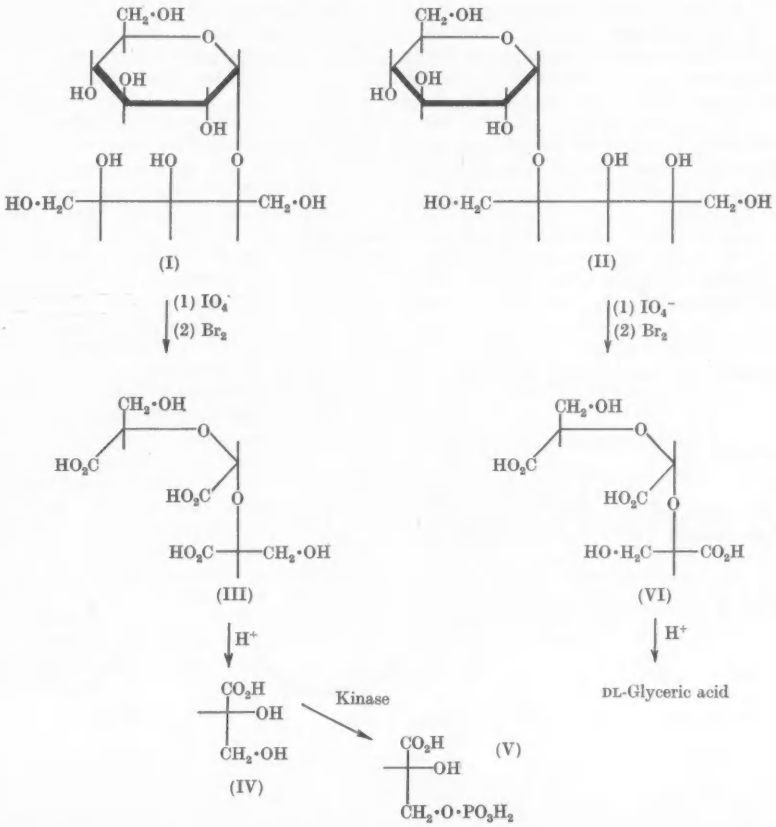
The two possible structures (I and II) for the glucosylribitol can be distinguished from each other by considering the stereochemistry of their oxidation products.

The structure (I), on oxidation with periodate, then with bromine, would give the tricarboxylic

acid (III); this would yield on acid hydrolysis 2 mol.prop. of D-glyceric acid (IX). On the other hand, the alternative structure (II) would give the tricarboxylic acid (VI), which would yield on acid hydrolysis 2 mol.prop. of DL-glyceric acid. In a similar degradation of the glucosylribitol from *B. subtilis* teichoic acid, the configuration of the glyceric acid was determined from its optical rotation in the presence of molybdate, but in the present work an alternative procedure was adopted. The glyceric acid was isolated from the reaction mixture by paper chromatography, eluted from the paper and then determined chemically by oxidation of a sample with sodium metaperiodate. Its configuration was established by phosphorylation with adenosine triphosphate in the presence of crude yeast hexokinase to give D-glyceric acid 3-phosphate (V). This was determined by its ability to oxidize reduced diphosphopyridine nucleotide in the presence of a rabbit-muscle-enzyme preparation. The enzymic method for determining D-glyceric acid 3-phosphate is similar to that used previously for establishing the configuration of the ribitol phosphate residue in cytidine diphosphate ribitol, and is known to be specific for the D-series (Baddiley, Buchanan & Carss, 1957*a*).

When the glucosylribitol from *L. arabinosus* teichoic acid was subjected to this procedure, the resulting glyceric acid, after phosphorylation, oxidized 1·9 mol.prop. of pyridine nucleotide: authentic D-glyceric acid, and the glyceric acid obtained from α-methyl glucoside, oxidized 2 mol.-prop. of pyridine nucleotide; DL-glyceric acid oxidized 1 mol.prop. of nucleotide. Thus, the glyceric acid from the glucosylribitol must have the D-configuration and the glucosylribitol is correctly represented as 4-*O*-(α-D-glucopyranosyl)-D-ribitol (I).

This combined chemical and enzymic method for determining the structure (I) of the glucosylribitol



is conveniently carried out on a small scale (3–4 μmoles of glycoside), and could be applied to the determination of the stereochemical series of many other glycosides. It has been used successfully here for determining the stereochemical series of the glucose in α-methyl D-glucoside and, with minor modification, has been applied in other work on the structure of nucleotides containing D-glucose or D-mannose (Blumsom & Baddiley, 1961). It is likely that this procedure will have considerable general use in the carbohydrate field.

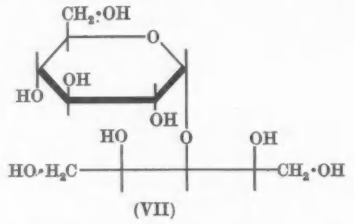
After several chromatographic separations, a pure specimen (1·7 mg.) of an isomeric glucosylribitol was obtained from a teichoic acid hydrolysate. This was non-reducing and on acid hydrolysis gave glucose, ribitol and anhydroribitol. Equimolar amounts of glucose and anhydroribitol were formed on vigorous acid treatment. The optical rotation of the glucosylribitol ($[\alpha]_D^{22} + 78 \pm 20°$) suggests that the glucosyl linkage is α, and this is supported by the stability of this glucoside in the presence of a β-glucosidase.

On oxidation of the isomeric glucosylribitol with sodium metaperiodate in dilute solution in the dark at room temperature, 4–5 mol.prop. of periodate were consumed and 2 mol.prop. of formaldehyde were produced. It follows that the glucose is attached to the hydroxyl at position 3 on the ribitol. Confirmation of the structure 3-*O*-(α-D-glucopyranosyl)ribitol (VII) was obtained by oxidation at pH 8 with a large excess of sodium metaperiodate at 37°. Hough & Perry (1956) have shown that under these conditions compounds that would be expected to be oxidized to derivatives of malondialdehyde are readily susceptible to a further degradation known as 'over-oxidation'. In a glucosylribitol this could only occur if the glucosyl residue is at the 3-position. When the glucoside was oxidized in this way, 3 mol.prop. of formaldehyde was produced, showing that 'over-oxidation' had occurred and therefore that the glucose was attached to the hydroxyl at the 3-position of the ribitol.

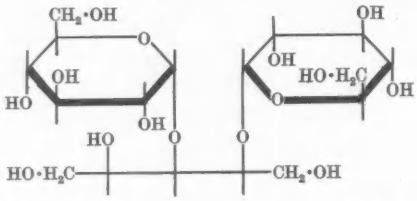
This structure was supported by the characteristic yellow colour given by the compound on paper chromatograms sprayed with the periodate–Schiff reagents. This behaviour has been observed previously with 3-substituted ribitol derivatives (Baddiley, Buchanan & Carss, 1957*b*).

The diglucosylribitol was precipitated from aqueous ethanol as a white deliquescent powder, which was probably microcrystalline. Vigorous acid hydrolysis gave glucose and anhydroribitol in the molar ratio 2:1. The high positive optical rotation indicated that both glucose units are present as α-glucosyl residues; this conclusion was supported by the failure of a β-glucosidase to hydrolyse the glycoside.

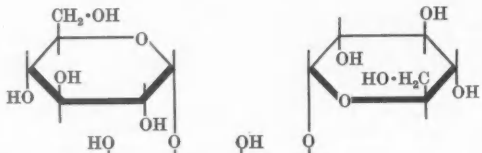
The diglucosylribitol consumed 5 mol.prop. of periodate, giving 2 mol.prop. of formic acid and 1 mol.prop. of formaldehyde. Reduction of the oxidation products with borohydride, then acid hydrolysis, gave glycerol and erythritol. Careful acid hydrolysis of the diglucoside gave glucose and two isomeric non-reducing monoglucosides of ribitol. It follows that the two glucose residues are each directly attached to the ribitol and cannot be present in a glucosylglucose unit.



(VII)



(VIII)



(IX)

From the above evidence it follows that the two glucose residues in the diglucoside must occupy positions 3 and either 4 (2) or 5 (1) of the ribitol. The 3:4- and 3:5-diglucosyl-D-ribitol structures are shown in (VIII) and (IX) respectively.

The isomeric structures were distinguished from each other by studies on the monoglucosides obtained by careful acid hydrolysis. The two monoglucosides were separated from each other by paper chromatography, and both gave equimolar amounts of glucose and anhydroribitol on vigorous treatment with acid. One was identified as 3-*O*-(α-D-glucospyranosyl)ribitol (VII) by paper-chromatographic comparison with the 3-glucosylribitol isolated previously. It also gave the characteristic yellow colour with the periodate–Schiff reagents, and on periodate oxidation at pH 8, 'over-oxidation' occurred, 3 mol.prop. of formaldehyde being formed.

The other glucosylribitol from the diglucoside was indistinguishable on paper chromatograms and in colour reactions from 4-*O*-(α-D-glucopyranosyl)-D-ribitol (I). On oxidation with periodate, followed by reduction with borohydride and acid hydrolysis, it gave glycerol but neither erythritol nor ethylene glycol. The 5(1)-glucosylribitol which would be formed by partial hydrolysis of structure (IX) would give ethylene glycol by this procedure; thus, structure (IX) is untenable for the diglucoside. Moreover, the aldehyde obtained by oxidizing the diglucoside with periodate is particularly labile towards alkali. This can be explained readily on the basis of a 3:4 (2) structure as in (VIII).

The structure (VIII) was distinguished from the isomeric 2:3-di-*O*-(α-D-glucopyranosyl)-D-ribitol by stereochemical analysis of the 4(2)-glucosylribitol obtained by partial hydrolysis of the diglucoside. The glyceric acid obtained by oxidizing the glucosylribitol with periodate then bromine followed by hydrolysis had the D-configuration. Consequently, the glucosylribitol must be the 4-glucoside (I), and the diglucosylribitol must be correctly represented as 3:4-di-*O*-(α-D-glucopyranosyl)-D-ribitol (VIII).

Although the glucosylribitol (I) and the diglucosylribitol (VIII) are the principal components of the teichoic acid from *L. arabinosus* walls, their relative amounts and the relative amounts of ribitol and isomeric glycosides vary in different samples of teichoic acid.

SUMMARY

1. It is confirmed that the teichoic acid from the walls of *Lactobacillus arabinosus* is composed of ribitol, phosphate, D-glucose and D-alanine.

2. Hydrolysis in alkali, followed by removal of phosphate from the products with a phosphatase, gave mainly a mono- and a di-glucosylribitol. Small amounts of anhydroribitol and an isomeric monoglucosylribitol are also formed, and occasionally traces of an isomeric diglucosylribitol have been detected. Teichoic acid preparations from some batches of micro-organisms but not others yield appreciable amounts of ribitol.

3. The principal monoglucosylribitol has the structure 4-*O*-(α-D-glucopyranosyl)-D-ribitol (I). This has been established by studies on optical rotation, hydrolysis, periodate oxidation and the stereochemistry of degradation products.

4. A general method is described for determining the stereochemical series of sugars from their glycosides on a microscale. This depends upon oxidation of the glycoside with periodate then bromine, hydrolysing the product with acid, and determining the configuration of the resulting glyceric acid by enzymic phosphorylation then oxidation of reduced diphosphopyridine nucleotide in the presence of a rabbit-muscle preparation.

5. The monoglucosylribitol formed in small amounts is 3-*O*-(α-D-glucopyranosyl)ribitol (VII). The structure of this compound was established by methods similar to those adopted for the isomeric 4-glucoside.

6. The diglucoside is 3:4-di-*O*-(α-D-glucopyranosyl)-D-ribitol (VIII). On careful acid hydrolysis it yields glucose and a mixture of 3- and 4-glucosylribitol identical with those isolated before.

7. The teichoic acid from the walls of *Lactobacillus arabinosus* differs markedly from the other ribitol teichoic acid which has been studied in detail, i.e. that from *Bacillus subtilis*, in that both mono- and di-glucoside residues are present and the glycosidic linkages all have the α-configuration.

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Optical Rotation of Soluble Feather Keratin

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A new structure has recently been proposed for feather keratin by Ramachandran & Dweltz (1961), which is based on a collagen-like triple helix and which is in good agreement with its detailed X-ray diffraction pattern. In support of this, they showed that feather keratin and collagen exhibit remarkable resemblances in their infrared-absorption spectra and birefringence, in addition to the nature of oriented crystallization of inorganic salts on the two fibrous proteins and in their behaviour on treatment with chemical reagents. In the present paper details are given of the measurement of the optical rotation of soluble feather keratin, which again is found to be close to that of collagen both in sign and magnitude.

MATERIALS AND METHODS

White goose-feather keratin was used in these experiments. The samples were first thoroughly degreased by washing in a number of changes of benzene, then cleaned by washing in a number of changes of distilled water and subsequently air-dried.

The solution of soluble SH-keratin of feather was prepared according to the method of Woodin (1954, 1956). A weighed amount (about 1 g.) of dry feathers was taken and the extraction made in a 50 ml. mixture of 10 M-urea, 0·1 M-$NaHSO_3$ and 0·05 M-sodium phosphate buffer at a pH of about 8·5 for 48 hr. at 60°. The extract was cooled to room temperature and filtered, and the clear protein solution collected. This was then made up to 50 ml. with the urea mixture and its optical rotation measured accurately at 25° with a Hilger standard polarimeter.

The protein concentration of feather keratin in solution was determined from the non-diffusible N content. For this purpose 4 ml. portions of the urea–bisulphite–protein solution were pipetted into cellophan sacs and dialysed against running tap water for various periods ranging from 1 to 7 days in steps of 1 day each. After the fourth day the estimated N was constant, but decreasing very slowly, probably because a fraction of the protein content was also diffusing out of the cellophan sac. A correction for this was made from the rate of change of concentration observed from the fourth to the seventh day.

The estimation of the N content in each case was carried out as follows: The contents of each dialysis sac was quantitatively transferred after dialysis to a 50 ml. flask and digested with conc. H_2SO_4 (A.R.) for 3 hr., over a microburner. Reaction was completed by adding 1–2 ml. of 30% H_2O_2 so as to remove any faint-yellow colour produced during digestion. The digest was then cooled in an ice bath and neutralized with NaOH (A.R.) added in drops, to a pH of approx. 7, and then made up to 100 ml. The N in this neutralized digest was estimated by nesslerization. The colorimetric measurements were made in a Klett–Summerson photoelectric colorimeter by using a no. 54

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green filter with maximum transmittance in the range 540–600 mμ. The amount of N in each sample was obtained by comparison with a standard. The corresponding protein concentration was calculated, taking the N content obtained to be 16·5 % of the dry solid feather keratin originally dissolved (Woodin, 1954).

RESULTS AND DISCUSSION

The specific rotation, $[\alpha]_D^{25}$, of soluble feather keratin was $-430°$ at 25°. The estimated probable error is $\pm 45°$. This was for a solution of protein concentration 3 mg./ml. The result of these experiments therefore clearly shows that the solution of soluble feather keratin has a large negative specific rotation that is of the same order of magnitude as that of collagen. The specific rotation of collagen (ichthyocol) in citrate buffer at 10°, $[\alpha]_D^{10}$, has been found by Cohen (1955) to be $-350° \pm 30°$. In general both collagen and the triple helical polypeptides poly-L-proline and poly-L-hydroxyproline exhibit a large negative specific rotation, $[\alpha]_D$, being in the range of $-300°$ to $-400°$ (Sasisekharan, 1961). On the other hand polypeptides of the α-helical form generally have a specific rotation, $[\alpha]_D$, in the range 0–50°, whereas for wool (α-keratin) $[\alpha]_D$ is of the order of $-50°$ to 0°. In general, α-type proteins have a small negative rotation, whereas in the random-coiled or denatured form they have a specific rotation of the order of $-100°$. The fact that feather keratin exhibits a large negative rotation of $-430°$ provides further evidence that it has a structure similar to that of collagen. The optical-activity data also support the triple helical structure of feather keratin.

SUMMARY

1. The optical rotation of soluble feather keratin has been studied, and the specific rotation of the protein in solution at 25°, $[\alpha]_D^{25}$, has been found to be $-430°$. This value is very close to that observed for soluble collagen and the triple helical polypeptides, and very different from that observed for α-proteins. It thus supports the triple helical structure put forward for feather keratin from X-ray data by Ramachandran & Dweltz (1961).

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Insulin and Incorporation of Amino Acids into Protein of Muscle

CELLULAR AMINO ACID LEVELS AND AMINO*ISO*BUTYRIC ACID UPTAKE

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Kipnis & Noall (1958) found that insulin *in vitro* enhances the accumulation of a non-utilizable amino acid, α-amino*iso*butyric acid, by isolated rat diaphragm. This observation has led to the belief that insulin may bring about the stimulation of incorporation of amino acids into protein of diaphragm (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958*a*; Wool & Krahl, 1959*a*) by a promotion of entry of amino acids into the tissue. However, in opposition to this view, no significant effect of insulin on the accumulation of naturally occurring amino acids has been observed and there is substantial evidence that insulin can stimulate incorporation of amino acids into protein under conditions in which it is not affecting their transfer into the cell (Wool & Krahl, 1959*b*; Manchester & Krahl, 1959; Manchester & Young, 1960). The possibility has been put forward (Manchester, 1960, 1961; Tepperman & Tepperman, 1960) that the enhanced uptake of amino*iso*butyric acid in the presence of insulin arises as a consequence of an increased rate of protein synthesis and that the amino*iso*butyric acid enters the cell to take the place of the extra amino acids incorporated into protein. This possibility rests on the tacit assumption that an in-

creased rate of incorporation of labelled amino acids into protein does indeed represent an increased rate of protein synthesis. But, on the evidence at present available, an increased incorporation of labelled amino acids into protein of diaphragm could equally arise if insulin were merely to facilitate the access of amino acids from the medium to the site of protein synthesis, without changing the total amount of protein formed.

The purpose of this paper is to provide evidence to the effect that (in stimulating amino acid incorporation insulin does increase the amount of protein synthesized) and to discuss experiments designed to investigate the mechanism by which insulin enhances the uptake of amino*iso*butyric acid.

METHODS

Materials. Diaphragm was taken from female albino Wistar rats, 100–120 g., fed on a stock laboratory diet (Bruce & Parkes, 1949) and allowed free access to food and water at all times. The hemidiaphragms were incubated in Krebs bicarbonate buffer (Krebs & Henseleit, 1932), gassed with O_2+CO_2 (95:5), pH 7·4 at 37°. The buffer contained no glucose or other additions unless otherwise stated.

The insulin used (Boots Pure Drug Co. Ltd.) was a six-fold recrystallized sample of potency of about 20 units/mg. It was dissolved in N/300-HCl to a concentration of 20 units/ml. and diluted with buffer as required. Alkali-inactivated insulin was prepared as described by Sutherland & Cori (1948). Insulin (1 mg.) was heated at 37° for 2·5 hr. in 1 ml. of 0·08N-KOH. It was subsequently diluted with buffer to the required concentration. The effectiveness of the inactivation was judged by the inability of the sample to stimulate either amino acid incorporation or glucose uptake by isolated diaphragm. The serum albumin was a highly purified specimen from rat plasma, kindly provided by Dr P. N. Campbell (Courtauld Institute of Biochemistry, Middlesex Hospital, London). It had been separated from α_1-globulin by electrophoresis on cellulose acetate. Casein was a commercial preparation (British Drug Houses Ltd.). Tolbutamide (*N*-butyl-*N'*-toluene-*p*-sulphonylurea) was from Burroughs Wellcome and Co.; 1:2-dichloro-4-(*p*-nitrobenzenesulphonylamido)-5-nitrobenzene was kindly provided by Professor E. F. Gale (Department of Biochemistry, University of Cambridge). Proflavin (as the hemisulphate) was from British Drug Houses Ltd. α-Amino*iso*[1-^{14}C]butyric acid, [1-^{14}C]glycine and [8-^{14}C]adenine were obtained from The Radiochemical Centre, Amersham, Bucks.

Measurement of release of amino acids. In two beakers each containing 20 ml. of buffer at 0° were collected hemidiaphragms from six rats, each beaker containing a hemidiaphragm from each rat. After standing for a few minutes at 0° the six hemidiaphragms from each beaker were blotted and transferred to a 50 ml. conical flask in which was 6 ml. of buffer containing the additions stated in Tables 1–3. The second flask in each experiment acted as the control for the first. The flasks were quickly gassed with O_2+CO_2, sealed and incubated at 37° with shaking in a water bath. Samples (0·2 ml.) of medium were taken from each flask at 15, 30, 60 and 90 min., the flasks being quickly gassed after the taking of each sample. At 120 min. a final sample of medium was taken, the six hemidiaphragms from each flask were weighed (about 600 mg.) and placed in 10 ml. of water in a boiling tube. Each tube was placed in a boiling-water bath for 5 min., with occasional shaking, then cooled and a sample (0·2 ml.) taken. The samples of medium and tissue extract, as well as samples of buffer in which the diaphragms were soaked before incubation, a blank and a sample of a solution of valine containing 21 μg. of amino nitrogen/ml., were deproteinized by the addition of 3·5 ml. of N/70-H_2SO_4 followed by 0·3 ml. of 0·67% sodium tungstate soln. After the solutions had stood for 1 hr. any slight precipitate was removed by centrifuging, and a sample (2 ml.) from each taken for the amino acid estimation. For the results in Table 4 single hemidiaphragms were incubated for 2 hr., each in 1 ml. of medium. After incubation each hemidiaphragm was weighed and heated for 5 min. in a boiling-water bath in 2 ml. of water. Samples (0·2 ml.) of extract and medium were taken and deproteinized as described above.

The method for the estimation of amino acids was essentially that of Chibnall, Mangan & Rees (1958). To each sample was added 1 ml. of a buffer made up of 23 ml. of 5M-sodium acetate (pH 5·5), 2 ml. of 0·01M-KCN and 75 ml. of 2-methoxyethanol (methyl Cellosolve; Hopkin and Williams Ltd.). Ninhydrin (0·2 ml. of 10%, w/v, solution in 2-methoxyethanol) was then added and the samples were heated in a boiling-water bath for 20 min. They were then cooled, diluted to 10 ml. with 60% ethanol and the light-absorption at 570 mμ was measured in a spectrophotometer. Experience showed that only small quantities of amino acids were liberated during the initial soaking. These quantities have been left out of all calculations.

Measurement of the accumulation of aminoisobutyric acid. Measurement of the accumulation of α-amino*iso*[^{14}C]-butyric acid was carried out as described by Manchester & Young (1960). Hemidiaphragms (four per flask, in which was 4 ml. of buffer containing amino*iso*butyric acid at a concentration of 0·22 μmole/ml. and specific activity of about 1·8 μc/μmole, except where otherwise stated: Table 7) were incubated for 1·5 hr. at 37°. At the end of this period each hemidiaphragm was blotted, weighed and placed separately in 2 ml. of water. The tubes containing hemidiaphragms were heated for 5 min. in a boiling-water bath and then cooled. A sample (0·5 ml.) of medium was diluted to 5 ml. with water. Samples (1 ml.) of tissue extract and diluted medium were pipetted on to 2 cm. diam. aluminium disks, previously painted with a metal lacquer, and the liquid was removed by evaporation under an infrared lamp. The content of radioactivity of the disks was assessed in a Panex counter (Model D-554). Counts ranged between 500 and 3000/min.

Measurement of incorporation of [^{14}C]*glycine into protein.* The method for the measurement of incorporation of [^{14}C]glycine into protein was very similar to that described by Manchester & Young (1958*a*). Glycine was added to the medium at a concentration of 1 μmole/ml. (specific activity about 80 μc/m-mole) unless otherwise stated (Table 8). After incubation for 2 hr. each hemidiaphragm was first heated for 5 min. and then homogenized in about 10 ml. of 10% trichloroacetic acid. The precipitate was spun down and resuspended in about 10 ml. of 10% trichloroacetic acid and heated for 15 min. at 90°. The insoluble material was

collected and dissolved in 1·5 ml. of 0·4N-NaOH and reprecipitated by addition of 10% trichloroacetic acid. It was washed three times with acetone and finally suspended in a small quantity of ether and poured on to a tared aluminium disk, area about 7·5 cm.². The disks were allowed to dry, then weighed, and the content of radioactivity was assessed in a Nuclear-Chicago gas-flow counter (model C-110B). Each protein sample weighed about 15 mg. Since the sample weights were fairly constant no corrections for self-absorption were made in calculating specific activities.

Measurement of incorporation of [^{14}C]adenine into nucleic acid. [^{14}C]Adenine was added to the medium at a concentration of 0·01 μmole/ml. (specific activity about 5 μc/μmole). After incubation for 2 hr. each hemidiaphragm was homogenized in about 10 ml. of cold 10% trichloroacetic acid containing 0·1% of [^{12}C]adenine. The insoluble material was washed three times with cold 10% trichloroacetic acid containing [^{12}C]adenine, three times with acetone and poured in ether on to tared disks as described above.

Measurement of release of potassium. Release of potassium was determined by flame photometry as described by Randle & Smith (1958).

Measurement of uptake of glucose. Uptake of glucose was calculated from the disappearance of glucose from the medium. After incubation the diaphragm was blotted and weighed and the residual glucose in the medium determined colorimetrically by Somogyi's modification of Nelson's method (Somogyi, 1945).

RESULTS

Effect of insulin on amino acid concentrations in diaphragm

Kline (1949) found that during incubation of isolated rat diaphragm there was a release of amino acids into the medium. If insulin stimulates incorporation of amino acids by increasing the rate of protein synthesis, then in the presence of the hormone the rate of accumulation of amino acids in the medium should be decreased, either because the rate of exit declines or because a greater uptake of amino acids is required to sustain the increased rate of protein synthesis.

Release of free amino acids by isolated rat diaphragm during incubation was found to follow the pattern described by Kline (1949), about 40% of the amino acids released during the incubation period of 2 hr. appearing in the medium in the first 15 min. (Table 1). Addition of insulin to the medium produced a small decrease in the amount of amino acids released and, moreover, the absolute size of the insulin effect increased with time (Table 1). Addition of alkali-inactivated insulin, highly purified serum albumin, casein or glucose did not have any consistent effect on the release of amino acids by isolated diaphragm (Table 1).

Table 1. *Effect of insulin, various proteins and glucose on the release of amino acids by isolated rat diaphragm during incubation*

For details see Methods.

	Amino acid released (μg. of amino nitrogen/g. of tissue incubated)				
Incubation time (min.)	15	30	60	90	120
Additions to the medium					
1.* No addition	146	186	251	302	355
Insulin [5 μg. (0·1 unit)/ml.]	139	172	222	268	310
Decrease produced by insulin	7	14	29	34	45
(Range)	(3–11)	(10–20)	(15–40)	(29–44)	(29–57)
2. No addition	132	183	246	310	363
Inactivated insulin (5 μg./ml.)	136	183	259	307	358
Decrease produced by inactivated insulin	−4	0	−13	3	5
(Range)	(−9–0)	(−14–18)	(−25–7)	(−27–23)	(−20–21)
3. No addition	155	186	259	316	365
Serum albumin (100 μg./ml.)	156	186	257	298	357
Decrease produced by albumin	−1	0	2	18	8
(Range)	(−10–7)	(−5–5)	(−1–5)	(10–26)	(1–15)
4. No addition	167	224	290	357	418
Casein (250 μg./ml.)	156	210	275	349	410
Decrease produced by casein	11	14	15	8	8
(Range)	(5–16)	(−3–26)	(5–25)	(−11–27)	(−24–39)
5. No addition	167	223	290	341	390
Glucose (2·5 mg./ml.)	170	229	286	354	387
Decrease produced by glucose	−3	−6	4	−13	3
(Range)	(−4 to −2)	(−17–5)	(−13–21)	(−20 to −6)	(−36–42)

* 1 and 2 are each the mean of three experiments, each involving six pairs of hemidiaphragms; 3, 4 and 5 are each the mean of two experiments.

Although the diminution of release of amino acids brought about by insulin was small, in each experiment the difference was remarkably consistent and decisive by comparison with the figures obtained with the inactivated insulin, serum albumin, casein or glucose. A significant diminution in the release of amino acids after 2 hr. of incubation in the presence of insulin is shown in Table 4.

Since the release of amino acids during the first 15 min. of incubation may arise largely as the result of damage sustained by the tissue during excision, Kline (1949), who was using the release of amino acids from isolated diaphragm as a measure of the rate of protein catabolism, based his calculations on the rate of release of amino acids after 15 min. It will be seen (Table 2) that, when from the results in Table 1 the rate of release of amino acids by diaphragm is calculated for periods after the first 15 min., a diminution in the presence of insulin is again apparent. As in Table 1, no consistent effect of addition of inactivated insulin, serum albumin, casein or glucose is observable in Table 2.

The suppression by insulin of the release of amino acids by diaphragm during incubation does not result from an increased retention of amino acids in the cell pool, for higher concentrations of amino acids in diaphragm were not observed in the

Table 2. *Effect of insulin, various proteins and glucose on the release of amino acids by isolated rat diaphragm during incubation after the first 15 min. of incubation*

	Amino acids released (μg. of amino nitrogen/g. of tissue incubated) after further incubation, less that released in the first 15 min. of incubation			
Additional incubation time (min.)	15	45	75	105
Additions to the medium				
1. No addition	40	105	156	209
Insulin [5 μg. (0·1 unit)/ml.]	33	83	129	171
Decrease produced by insulin	7	22	27	38
2. No addition	51	114	178	231
Inactivated insulin (5 μg./ml.)	47	123	171	222
Decreased produced by inactivated insulin	4	−9	7	9
3. No addition	31	104	161	210
Serum albumin (100 μg./ml.)	30	101	142	201
Decrease produced by albumin	1	3	19	9
4. No addition	57	122	190	251
Casein (250 μg./ml.)	54	118	193	254
Decrease produced by casein	3	4	−3	−3
5. No addition	56	123	174	223
Glucose (2·5 mg./ml.)	59	116	184	217
Decrease produced by glucose	−3	7	−10	6

Table 3. *Effect of insulin, various proteins and glucose on the quantity of free amino acids found in isolated rat diaphragm and the incubation medium after incubation for 2 hr.*

	Amino acids found (μg. of amino nitrogen/g. of tissue incubated)			Decrease (%)
Additions to the medium	In tissue	In medium	Total	
1.* No addition	430	355	785	11·7
Insulin [5 μg. (0·1 unit)/ml.]	383	310	693	
2. No addition	271	363	634	−1·1
Inactivated insulin (5 μg./ml.)	283	358	641	
3. No addition	371	365	736	−1·0
Serum albumin (100 μg./ml.)	386	357	743	
4. No addition	272	418	690	0·5
Casein (250 μg./ml.)	277	410	687	
5. No addition	390	371	761	1·2
Glucose (2·5 mg./ml.)	387	365	752	

* 1 and 2 are each the mean of three experiments, each involving six pairs of hemidiaphragms; 3, 4 and 5 are each the mean of two experiments.

presence of the hormone (Table 3). Rather, the amount of amino acids in the tissue was less when it was incubated for 2 hr. in the presence of insulin than when the insulin was omitted (Table 3). Addition of inactivated insulin, serum albumin, casein or glucose had no similar effect (Table 3). When the amount of amino acids in the medium and that remaining in the tissue after incubation for 2 hr. are added together it is found that the quantity of amino acids in the system is significantly less in the presence of insulin than in its absence (Tables 3 and 4). No comparable changes were produced by inactivated insulin, serum albumin, casein or glucose (Table 3). Thus insulin can not only depress the accumulation of amino acids in the medium, but also diminishes the amount of amino acids in the system as a whole (those in the tissue and the medium added together).

Table 4. *Effect of insulin on the quantity of free amino acids found in isolated rat diaphragm and the incubation medium after incubation*

Similar to Table 3, no. 1, except that each hemidiaphragm was incubated separately for 2 hr. in 1 ml. of medium in order that sufficient observations might be obtained for statistical analysis. Each figure is the mean ±S.E. of the mean of eight observations. The value of P for a difference which is significant is indicated.

Addition to the medium	Amino acids found (μg. of amino nitrogen/g. of tissue incubated)				
	In tissue	In medium		Total	
No addition	429±15	405±15	$P < 0.05$	834± 9	$P < 0.01$
Insulin [5 μg. (0·1 unit)/ml.]	422±14	354±16		776±17	

Table 5. *Effect of insulin, various proteins and glucose on the ratio of concentration of amino acids within isolated diaphragm to that in the medium after incubation for 2 hr.*

	Concn. of amino acids without additions			Concn. of amino acids with additions shown		
	In tissue (μg. of amino nitrogen/g. of tissue water*)	In medium (μg. of amino nitrogen/ml. of medium)	Ratio	In tissue (μg. of amino nitrogen/g. of tissue water*)	In medium (μg. of amino nitrogen/ml. of medium)	Ratio
				With insulin [5 μg. (0·1 unit)/ml.]		
Expt. 1	747	46·0	16·2	558	42·8	13·0
Expt. 2	509	46·3	11·0	504	37·3	13·5
Expt. 3	467	37·1	12·6	466	30·7	15·2
		Average	13·3		Average	13·9
				With inactivated insulin (5 μg./ml.)		
Expt. 1	313	40·4	7·8	367	36·5	10·0
Expt. 2	346	39·7	8·7	325	36·7	8·8
Expt. 3	425	44·6	9·5	440	43·6	10·1
		Average	8·7		Average	9·6
				With serum albumin (100 μg./ml.)		
Expt. 1	423	39·8	10·6	448	39·5	11·3
Expt. 2	320	36·2	8·8	325	37·4	8·7
		Average	9·7		Average	10·0
				With casein (250 μg./ml.)		
Expt. 1	357	39·4	9·1	380	38·4	10·1
Expt. 2	369	37·5	9·8	357	35·8	10·0
		Average	9·5		Average	10·0
				With glucose (2·5 mg./ml.)		
Expt. 1	399	40·3	9·9	384	42·5	9·0
Expt. 2	343	42·4	8·1	347	40·9	8·5
		Average	9·0		Average	8·8

* Tissue water has been assumed to be 75% of wet weight.

Since the quantity of amino acids found in the tissue is not raised in the presence of insulin, the ratio of the concentration of amino acids in the tissue to that in the medium therefore remains unchanged. The ratios shown in Table 5, although they vary considerably from one experiment to another, do not provide any evidence to suggest that insulin promotes the accumulation of the naturally occurring amino acids and ninhydrin-reacting peptides of diaphragm.

The release of amino acids by isolated diaphragm is generally thought of as a measure of the extent to which protein breakdown exceeds protein synthesis in this preparation. However, as the figures in Table 6 indicate, the major part of the amino acids appearing in the medium during incubation represents the diminution of the pool of free amino acids in the tissue, the cell pool of free amino acids in isolated hemidiaphragm undergoing a considerable reduction in size. Thus 80% of the amino nitrogen content of the system at the end of the incubation was present as free amino nitrogen at the beginning (Table 6).

Effect of insulin on accumulation of aminoisobutyric acid

It has been suggested (Manchester, 1960, 1961; Tepperman & Tepperman, 1960) that the increased accumulation of α-amino*iso*butyric acid by diaphragm observed in the presence of insulin arises, not from an action of insulin on the accumulation of amino acids, but as a consequence of the acceleration by insulin of the rate of protein synthesis. This suggestion assumes that amino acids can be treated as a class of compounds which for accumulation purposes are capable of replacing and displacing each other. The work of Christensen and his colleagues (summarized in Christensen, 1960) supports this assumption. Because of the increased rate of utilization of amino acids from the cell pool for protein synthesis in the presence of insulin, the rate of release of amino acids declines and the rate of entry or re-entry increases in order to preserve a constant concentration gradient between the tissue and the medium. As α-amino-*iso*butyric acid is an amino acid it is subject to the same fluxes and movements as the other amino acids. Because amino*iso*butyric acid is not incorporated into protein it accumulates in the tissue and, by acting as the replacement for the extra amino acids incorporated into protein in the presence of insulin, so it is progressively accumulated over a period of time under the influence of the hormone.

If this hypothesis is correct it should be open to experimental verification in at least two ways. First, if a naturally occurring amino acid is added to the medium in large quantity then this amino acid rather than the trace quantities of amino*iso*-butyric acid present should make up the deficiency caused, in the presence of insulin, by the amino acids entering protein, and under these conditions an effect of insulin on the accumulation of amino-*iso*butyric acid would not be expected. As a corollary of this, if the quantity of amino*iso*butyric acid added to the medium is increased, then the quantity entering the tissue in the absence of insulin will rise and any extra amino acids incorporated into protein will become negligible in quantity by comparison with the basal uptake. Thus it is to be expected that, as the quantity of amino*iso*butyric acid added to the medium is increased, the size of the insulin effect will diminish, and this is in fact observed (Table 7). Increasing the concentration of amino*iso*butyric acid added to the medium from 0·2 to 20 μmoles/ml. progressively obscures an effect of insulin on its accumulation by diaphragm. By comparison, a significant stimulation of incorporation of [^{14}C]glycine into protein is observable at concentrations of glycine as great as 20 μmoles/ml. (Table 8).

In a study of the effect of the addition of other amino acids to the medium on the accumulation of amino*iso*butyric acid it was found (Table 9) that addition of L-valine, L-leucine, glycine and L-alanine at 20 mM concentration decreased very

Table 6. *Release of amino acids by isolated rat diaphragm during incubation*

Number of observations is shown in parentheses. Tissue values are means ± S.E. Release of amino acids into the medium by 479 mg. of hemidiaphragm was (21·5 × 6) = 129 μg. of amino nitrogen = 270 μg./g. of tissue.

Amino nitrogen content of tissue and medium before incubation = 452
Amino nitrogen content of tissue and medium after incubation = 282 + 270 = 552
Therefore increase in amino nitrogen content of the system during incubation = 100 μg./g. of tissue.

	Intact diaphragms	Hemidiaphragms
Content of diaphragm before incubation (μg. of amino nitrogen/g. of tissue) (6)	522 ± 9	452 ± 15
Content of diaphragm after incubation for 1·5 hr. (6)	455 ± 11	282 ± 9
Decrease	67	170
Content of medium after incubation (μg. of amino nitrogen/ml. of medium)	54·5	21·5
Ratio of internal concn. to external concn.	8·3	13·1

substantially the size of the effect of insulin on the accumulation of α-amino*iso*butyric acid. Leucine, glycine and alanine at 2 mM also had a considerable effect in this respect, although addition of valine at 2 mM concentration did not. On the other hand, even in the presence of 20 mM concentration of amino acid there was a significant, even if smaller, stimulation by insulin of the accumulation of amino*iso*butyric acid.

The second way in which experimental verification of the hypothesis advanced above has been sought is based on the argument that, under condi-

Table 7. *Effect of change of concentration of α-amino*iso[^{14}C]*butyric acid on the stimulation by insulin of its accumulation by isolated rat diaphragm*

Each figure is the mean ± S.E. of the mean of four observations. Value of P for a difference between means which is significant is indicated.

Concn. of amino*iso*butyric acid added to the medium (mM)	Ratio: Concn. of amino*iso*butyric acid in tissue water / Concn. of amino*iso*butyric acid in incubation medium			
	No added insulin	Insulin added (0·1 unit/ml.)	Difference	Significance of insulin effect (P)
0·22	2·04 ± 0·13 } $P < 0·01$	4·08 ± 0·11 } $P < 0·001$	2·04	< 0·001
2·0	1·45 ± 0·076 }	2·08 ± 0·12 }	0·63	< 0·01
20·0	1·08 ± 0·020 } $P < 0·01$	1·10 ± 0·024 } $P < 0·001$	0·02	—

Table 8. *Stimulation by insulin of the incorporation of* [^{14}C]*glycine at varying concentrations into protein of isolated rat diaphragm*

Each figure is the mean ± S.E. of the mean of six observations. The stimulation by insulin is in each case significant ($P < 0·01$). Quantity of radioactivity added to the medium was in each case the same (0·08 μc/ml.).

Concn. of glycine added to the medium (mM)	Radioactivity in diaphragm protein (counts/min./mg.)		Stimulation (%)
	No added insulin	Insulin added (0·1 unit/ml.)	
0·003	44·6 ± 2·22	82·2 ± 7·29	85
2	11·1 ± 0·66	16·1 ± 0·57	45
20	1·76 ± 0·10	2·62 ± 0·20	49

Table 9. *Effect of addition of other amino acids on the accumulation of α-amino*iso[^{14}C]*butyric acid by isolated rat diaphragm and the influence of insulin on this process*

Each figure is the mean ± S.E. of the mean of four observations.

Additions to the medium	Ratio: Concn. of amino*iso*butyric acid in tissue water / Concn. of amino*iso*butyric acid in incubation medium			Significance of insulin effect (P)
	No added insulin	Insulin added (0·1 unit/ml.)	Difference	
1. No addition	(*a*) 2·65 ± 0·09	5·35 ± 0·29	2·70	< 0·001
L-Valine (2 mM)	(*b*) 2·56 ± 0·08	5·00 ± 0·20	2·44	< 0·001
L-Valine (20 mM)	(*c*) 1·10 ± 0·08	1·78 ± 0·10	0·68	< 0·01
Significance of differences	(*a*) – (*c*) $P < 0·001$	(*a*) – (*c*) $P < 0·001$		
2. No addition	(*a*) 2·24 ± 0·06	4·31 ± 0·12	2·07	< 0·001
L-Leucine (2 mM)	(*b*) 2·20 ± 0·11	3·37 ± 0·12	1·17	< 0·001
L-Leucine (20 mM)	(*c*) 1·63 ± 0·06	2·49 ± 0·20	0·86	< 0·01
Significance of differences	(*a*) – (*c*) $P < 0·001$	(*a*) – (*b*) $P < 0·01$ (*a*) – (*c*) $P < 0·001$		
3. No addition	(*a*) 1·92 ± 0·06	4·08 ± 0·32	2·16	< 0·001
Glycine (2 mM)	(*b*) 1·70 ± 0·11	2·58 ± 0·34	0·88	< 0·05
Glycine (20 mM)	(*c*) 1·21 ± 0·04	1·64 ± 0·06	0·43	< 0·001
Significance of differences	(*a*) – (*c*) $P < 0·001$	(*a*) – (*b*) $P < 0·02$ (*a*) – (*c*) $P < 0·001$		
4. No addition	(*a*) 2·49 ± 0·19	6·27 ± 0·52	3·78	< 0·001
L-Alanine (2 mM)	(*b*) 1·56 ± 0·11	2·65 ± 0·27	1·09	= 0·01
L-Alanine (20 mM)	(*c*) 0·95 ± 0·024	1·09 ± 0·051	0·14	= 0·05
Significance of differences	(*a*) – (*b*) $P < 0·01$ (*a*) – (*c*) $P < 0·001$	(*a*) – (*b*) $P < 0·001$ (*a*) – (*c*) $P < 0·001$		

tions in which protein synthesis is prevented, there can be no effect of insulin in increasing the entry of amino acids into protein. Therefore, according to the hypothesis, no effect of insulin on the accumulation of amino*iso*butyric acid should be observable.

Of the various compounds available which might be expected to inhibit amino acid incorporation, general metabolic inhibitors (cyanide, azide, etc.) are not suitable for the present purpose, for although they effectively inhibit this process (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Manchester & Young, 1959) they also interfere with amino acid accumulation. Amino acid analogues might also be expected to interfere with amino acid accumulation and are therefore unsuitable potential inhibitors of protein synthesis (though neither DL-ethionine nor DL-norleucine at concentration of 20 mM has been found to decrease incorporation of [^{14}C]glycine). Unfortunately it has not proved possible to find any compound which will specifically inhibit amino acid incorporation without interfering with other metabolic activities and energy-producing reactions of the cell as judged by appearance, potassium release and glucose uptake (Randle & Smith, 1958). Neither chloramphenicol nor 8-azaguanine, which have been widely used as inhibitors of protein synthesis in bacterial systems, had any observable effect on amino acid incorporation into protein of diaphragm (Table 10). Since 8-azaguanine had no effect on incorporation of [^{14}C]adenine into nucleic acid either, it may be that it is unable to penetrate into diaphragm to the site of nucleic acid synthesis. 5-Fluoro-6-deoxyuracil, which has been found to interfere with synthesis of deoxyribonucleic acid (Flaks & Cohen, 1959), was without effect on amino acid incorporation in the short duration of these experiments (Table 10). Manchester & Young (1958*b*) found that tolbutamide inhibited amino acid incorporation, but at the concentrations required to observe this effect tolbutamide also stimulated release of potassium and uptake of glucose (Table 10). 1:2-Dichloro-4-(*p*-nitrobenzenesulphonylamido)-5-nitrobenzenes, which has been used by Woolley (1952) as a growth inhibitor, severely inhibited incorporation at 10 μM concentration and above, but, as with tolbutamide, at this concentration it also stimulated release of potassium and uptake of glucose (Table 10). Proflavin has long been known as a growth inhibitor in bacterial systems. At 20 μg./ml. it appeared to be inhibitory towards amino acid incorporation and to promote only a slight release of potassium. Since it is probable that the rate of amino acid incorporation in the presence of an inhibitor declines steadily throughout the first part of the incubation, in the subsequent experiments with proflavin (Table 11) diaphragm was first preincubated for 30 min. in buffer containing 20 μg. of proflavin/ml. before being transferred to the final incubation medium containing [^{14}C]glycine or amino*iso*[^{14}C]butyric acid, where the concentration of proflavin was 10 μg./ml. Under these conditions there was a significant release of potassium

Table 10. *Effect of addition to the medium of various compounds, which can under certain circumstances inhibit protein synthesis, on incorporation of amino acid into protein, uptake of glucose, release of potassium and incorporation of nucleotide into nucleic acid of isolated rat diaphragm*

—, No observation was made. 'Stimulation' implies a significant effect. DCDNS, 1:2-Dichloro-4-(*p*-nitrobenzenesulphonylamido)-5-nitrobenzene.

Additions to medium	Inhibition of incorporation of [^{14}C]glycine into protein (%)	Effect on uptake of glucose	Effect on release of potassium
Tolbutamide	83 (6)	Stimulation	Stimulation
Chloramphenicol (50 μg./ml.)	7 (6)	—	—
(250 μg./ml.)	18 (3)	—	—
8-Azaguanine (30 μg./ml.)	0 (6)*	—	—
(300 μg./ml.)	0 (3)*	—	—
DCDNS (100 μM)	92 (6)†	No change	—
(10 μM)	69 (6)	Stimulation	Stimulation
(1 μM)	3 (6)	—	—
5-Fluoro-6-deoxyuracil (10 μM)	7 (3)	—	—
Proflavin (100 μg./ml.)	90 (3)*	—	—
(20 μg./ml.)	62 (3)	—‡	Slight increase
(4 μg./ml.)	29 (3)	—	No effect

* In these experiments 8-azaguanine was tested also for any effect on incorporation of [^{14}C]adenine into nucleic acid (see Methods). It had no effect at either concentration.
† Severe rigor.
‡ See Table 11.

Table 11. *Effect of proflavin* in vitro* *on the incorporation of* [^{14}C]*glycine into protein, accumulation of* α*-amino*iso*butyric acid, release of potassium and uptake of glucose by isolated rat diaphragm*

Each figure, except for release of potassium marked (†), is the mean±S.E. of the mean of six observations.

	Glucose (2·5 mg./ml.) added to the medium	Length of final incubation (hr.)	Additions to the medium: Proflavin — No addition (*a*)	Proflavin — Insulin added (0·1 unit/ml.) (*b*)	Controls — No addition (*c*)	Controls — Insulin added (0·1 unit/ml.) (*d*)	Significance of difference (*P*)
1. Incorporation of [^{14}C]glycine (counts/min./mg.)	+	2·0	4·31±0·29		23·0±0·82		(*c*) − (*a*) < 0·001
Release of potassium (μ-equiv./g. wet wt.)			11·0±1·0		0·1±1·0		(*c*) − (*a*) < 0·001
Uptake of glucose (mg./g. wet wt./hr.)			4·23±0·21		2·46±0·32		(*a*) − (*c*) = 0·001
2. Incorporation of [^{14}C]glycine (counts/min./mg.)	−	2·0	3·45±0·21	5·20±0·38	21·8±1·17	38·4±1·70	(*b*) − (*a*) < 0·01 (*d*) − (*c*) < 0·001 (*c*) − (*a*) < 0·001
Release of potassium (μ-equiv./g. wet wt.)†			23	20	16	12	
3. Accumulation of α-amino*iso*butyric acid (concn. ratio)‡	−	1·5	2·23±0·09	3·32±0·24	2·65±0·15	6·33±0·35	(*b*) − (*a*) < 0·01 (*d*) − (*c*) < 0·001 (*d*) − (*b*) < 0·001 (*c*) − (*a*) < 0·05
Release of potassium (μ-equiv./g. wet wt.)†			24	11			

* The hemidiaphragms were preincubated at 37° for 30 min. in a medium containing 20 μg. of proflavin/ml., then transferred to the final medium which contained labelled materials±insulin. Incubation in this medium was for the period stated in the Table.

† Sum of potassium released during preincubation and final incubation (single pooled observations).

‡ Ratio: $\frac{\text{Concn. of amino}iso\text{butyric acid in tissue water}}{\text{Concn. of amino}iso\text{butyric acid in incubation medium}}$.

and increase of glucose uptake (Table 11), but the release of potassium was not as large as was found with the nitrobenzene compound or tolbutamide. Incorporation of glycine into protein was decreased by over 80% (Table 11). Although a significant stimulation of accumulation of amino*iso*butyric acid was still observable in the presence of proflavin, the size of the insulin effect was substantially less than that found with diaphragm without proflavin (Table 11). The amount of amino*iso*butyric acid accumulation in the absence of insulin showed a small reduction in the presence of proflavin.

DISCUSSION

Effect of insulin on the rate of protein synthesis

Although insulin consistently and significantly depressed the release of amino acids by diaphragm during incubation (Tables 1 and 4), the size of the insulin effect, roughly a 10% decrease, is much smaller than the magnitude of the effect of insulin on incorporation of labelled amino acids into the protein of diaphragm (50–100%: Manchester & Young, 1958*a*; Wool & Krahl, 1959*a*) normally observed. There are several possible reasons for this. First, if a portion of the release results from damage to the tissue sustained during excision, this will tend to obscure the size of the insulin effect. Thus when the quantity of amino acids released in the first 15 min. is disregarded (Table 2), the size of the insulin effect rises to 18%. Ideally, the intact diaphragm preparation of Kipnis & Cori (1957), whose cell pool of amino acids declines much less during incubation (Table 6), would be the more suitable system in which to measure the rate of release of amino acids as a result of protein catabolism, but with this preparation the concentration of amino acids in the medium rises to a higher value than with hemidiaphragms (Table 6), presumably because of the release of amino acids by the severed intercostal muscle. From the figure for the increase in the amino nitrogen content of the system during incubation (100 μg. of amino nitrogen/g. of tissue/1·5 hr.; Table 6) the excess of protein breakdown over protein synthesis in hemidiaphragms can be calculated as $100 \times 6{\cdot}25/1{\cdot}5 = 415\,\mu$g. of protein/g. of tissue/hr. Secondly, although the ninhydrin colour reaction is fairly specific for amino acids (Moore & Stein, 1954), ninhydrin does react with ammonia and with peptides such as carnosine, anserine and glutathione. Determination of residual ninhydrin-reacting material after evaporation to dryness at pH 10 indicated that only small quantities of ammonia are released by diaphragm. The presence of substantial quantities of di- and tri-peptides, however, whose movements it is assumed are not affected by insulin, might well be expected to mask the extent of the diminution by insulin of the release of amino acids when expressed as a percentage.

That insulin suppresses the rise in the total quantity of amino acids in the tissue and the medium (Tables 3 and 4) suggests that the hormone does bring about an increase in the amount of protein synthesized. It could be argued that a diminution in the rate of appearance of amino acids arises from a diminished rate of breakdown of protein rather than an increased rate of its synthesis, and it is not possible to say from the results whether insulin does or does not affect the rate of protein breakdown in diaphragm. [Opinion is divided about whether the rate of protein breakdown is altered in diabetes. Forker & Chaikoff (1952) concluded that the rate of protein catabolism of serum proteins was not markedly changed in diabetic dogs. Green & Miller (1960) found the rate of protein breakdown by the perfused diabetic liver to be elevated.] However, since amino acids incorporated into protein are not subsequently released on further incubation (Manchester, 1961), increased incorporation cannot result from decreased breakdown. In view of the stimulation by insulin of the incorporation of labelled amino acids into protein it seems very probable that at least part of the suppression of the appearance of amino acids must result from an increase in the rate of protein synthesis. From the figures in Table 3 the increase in the rate of protein synthesis brought about by insulin can be calculated as

$$(785 - 693) \times 6{\cdot}25 = 575\,\mu\text{g.}$$

of protein/g. of tissue/2 hr. If insulin stimulates the rate of protein synthesis by 50–100% then this gives a figure for the rate in the absence of added insulin of 575–1150 μg. of protein/g. of tissue/2 hr., which agrees well with a corresponding figure of 750 μg. estimated by Manchester, Randle & Young (1959) from figures obtained from the rate of incorporation of [^{14}C]glycine into protein of diaphragm.

The results included in Tables 1–4 are in conformity with and provide an understanding of the observation that insulin can suppress the rise of the blood amino acid concentration in the eviscerated animal (Mirsky, 1938; Frame & Russell, 1946; Ingle, Prestrud & Nezamis, 1947). As with the eviscerated animal (Frame & Russell, 1946; Ingle *et al.* 1947) and with the incorporation of labelled amino acids into protein of diaphragm (Manchester & Young, 1958*a*; Wool & Krahl, 1959*a*), the addition of glucose is without effect on the amounts of amino acids in the tissue and medium (Tables 1–5). The concentration ratios contained in Table 5 support the findings of

Manchester & Young (1960), Toyoda & Bessman (1960) and Guroff & Udenfriend (1961) that under the conditions in which insulin stimulates the accumulation of α-amino*iso*[^{14}C]butyric acid it has no effect on the accumulation of naturally occurring [^{14}C]amino acids. It is a curious circumstance, however, which is not at present explicable, that the concentration ratios obtained here are very much higher than those observed earlier with [^{14}C]amino acids, which were of the same order as those obtained with amino*iso*butyric acid in the absence of insulin (Manchester & Young, 1960). As Guroff & Udenfriend (1960) have recently shown for tyrosine, a [^{14}C]amino acid does not seem to equilibrate with more than a fraction of its own species contained in the cell pool.

Mechanism by which insulin enhances uptake of aminoisobutyric acid

Although the observation that addition of other amino acids depresses the effect of insulin on the accumulation of α-amino*iso*butyric acid (Table 9) is consistent with the hypothesis that its enhanced accumulation in the presence of the hormone results from the increased entry of other amino acids into protein, in so far as significant effects of insulin on the accumulation of amino*iso*butyric acid can be detected even in the presence of large concentrations of other amino acids the results could equally well be regarded as evidence inconsistent with this explanation. In the absence of insulin, as well as in its presence, addition of other amino acids depresses the accumulation of amino*iso*butyric acid, though this is marked only at the 20 mM concentration. It is not possible to say from these figures whether the latter results arise from displacement of one amino acid by another or from competition for the systems which transport amino acids into the cell. On the other hand, in support of the hypothesis advanced, there is a remarkable correlation between the extra amino*iso*butyric acid entering diaphragm in the presence of insulin and the extra amino acids incorporated into protein (see above). The average of the ratios for the accumulation of low concentrations of amino*iso*butyric acid in the absence of other added amino acids is (from Tables 7 and 9) 4·82 in the presence and 2·27 in the absence of insulin. The extra quantity of amino*iso*butyric acid accumulated in the presence of insulin in 1·5 hr. of incubation is therefore $(4{\cdot}82-2{\cdot}27)\times 0{\cdot}22 = 5{\cdot}6\,\mu$moles/ml. of tissue water = 4·2 μmoles/g. of tissue. Thus after 1·5 hr. the extra uptake of amino*iso*butyric acid in the presence of insulin is equivalent to about 420 μg. of amino acids/g. of tissue.

In the experiments with proflavin (Table 11) it is not possible to rule out completely the possibility that part of the reduction in the accumulation of amino*iso*butyric acid in the presence of insulin results from an interference by proflavin with the energy-producing operations of the cell. In fact the stimulation of release of potassium and uptake of glucose suggests that this does occur to some extent. Nevertheless, the potassium release seemed less severe and the inhibition of incorporation of glycine greater than with either 1:2-dichloro-4-(*p*-nitrobenzenesulphonylamido)-5-nitrobenzene or tolbutamide, and the uptake of amino*iso*butyric acid in the absence of insulin was only slightly reduced. Unless the mechanism by which these compounds interfere with protein synthesis is entirely due to an inhibition of energy-rich phosphate production, it is a curious coincidence that the point at which they begin to interfere with protein synthesis is at the same concentration as that at which they interfere with other metabolic activities. It is possible that protein synthesis in diaphragm muscle is too closely integrated with the overall metabolism of the cell for it to be possible to find anything which can specifically inhibit this process without producing a more general interference.

The results of Tables 7–11 thus do not provide unequivocal evidence in favour of the available hypothesis for the mechanism by which insulin stimulates accumulation of α-amino*iso*butyric acid. The findings that the effect of insulin on amino*iso*butyric acid accumulation is markedly decreased both when its own concentration is raised (Table 7) and in the presence of high concentrations of other amino acids (Table 9), and under conditions in which amino acid incorporation is severely inhibited (Table 11), are, however, precisely those to be expected on the basis of the suggested explanation. Manchester & Young (1960) found that under the conditions in which insulin stimulated the accumulation of amino*iso*-[^{14}C]butyric acid there did seem to be a smaller stimulation of accumulation of [^{14}C]glycine, though not of other [^{14}C]amino acids studied. Since the concentration of glycine in the cell pool is higher than for most other amino acids (unpublished observations), it is possible that glycine can under these circumstances act to some extent as though it were a non-utilizable amino acid. On the other hand, Guroff & Udenfriend (1961) found that insulin had no effect on the accumulation of α-methyltyrosine, an α-methyl amino acid like α-amino*iso*butyric acid, an observation which is not readily explicable on any basis at the present time.

Mechanism by which insulin stimulates protein synthesis

The precise mechanism by which insulin *in vitro* stimulates the incorporation of labelled amino acids

into protein of isolated tissues is at present unknown. Despite the pronounced stimulation by insulin of accumulation of amino*iso*butyric acid, the lack of observable enhancement of the accumulation of other naturally occurring amino acids (Manchester & Young, 1960; Toyoda & Bessman, 1960) and of α-methyltyrosine (Guroff & Udenfriend, 1961), and the stimulation by insulin of incorporation into protein of amino acids whose transfer into the cell has already taken place (Wool & Krahl, 1959*b*; Manchester & Krahl, 1959), argue against a direct effect of insulin on the transfer of amino acids from the medium into the cell pool. That an effect of insulin on incorporation of glycine is still detectable when the concentration of glycine is raised to as high a figure as 20 mM (Table 8) is an observation again consistent with this view, for under these conditions the figures obtained with amino*iso*butyric acid suggest that the rate of entry of amino acid into the cell is too large to be the limiting step.

Recent observations seem to indicate, however, that the pool of amino acids in diaphragm from which amino acids are taken for protein synthesis is not necessarily the same as the total amino acid pool of the tissue. Kipnis, Reiss & Helmreich (1961) have found that the time-curve for the incorporation of radioactivity into diaphragm protein is not what would be predicted from the rate of rise of specific activity of amino acids of the cell pool. Guroff & Udenfriend (1960) found that labelled tyrosine added to the medium exchanged with only a portion of the tyrosine already present in the pool of the diaphragm tissue. Thus the negative results of experiments designed to show an effect of insulin on the accumulation of amino acids under the conditions in which it stimulates accumulation of α-amino*iso*butyric acid (Manchester & Young, 1960; Toyoda & Bessman, 1960; Guroff & Udenfriend, 1961) do not necessarily preclude the possibility that insulin enhances the entry of amino acids into a much more limited pool. Although the increased rate of incorporation of labelled amino acids into protein seems to result from an increased rate of protein synthesis rather than merely a facilitation of the transfer of labelled amino acids from the medium to the pool from which amino acids required for protein synthesis are drawn, if the rate of protein synthesis were limited by the availability of amino acids in this pool, then an action of insulin to enhance their transfer there, either from the medium or from the other pool or pools, would bring about an increased rate of protein synthesis. Alternatively, as has been put forward elsewhere (Battaglia, Manchester & Randle, 1960; Tepperman & Tepperman, 1960), if insulin accelerates transport of glucose in muscle by restricting the access of energy-rich phosphate to a process concerned with the regulation of glucose transport (Randle & Smith, 1957), it may at the same time direct this energy-rich phosphate to the processes involved in protein synthesis whose rates of action are limited by the availability of energy-rich phosphate at this locus. At the present time it has not been possible to provide confirmation for either hypothesis.

SUMMARY

1. Insulin *in vitro* diminishes the rate of release of amino acids by isolated rat diaphragm during incubation. It does not affect the ratio of the distribution of amino acids between the tissue and the medium.

2. After incubation for 2 hr. the sum of the quantity of non-protein ninhydrin-reacting material contained in the tissue and that in the medium is less in the presence of insulin than in its absence. This observation is interpreted to mean that the enhanced rate of amino acid incorporation in the presence of insulin does represent an increased rate of synthesis of protein.

3. The possibility that the stimulation by insulin of accumulation of α-amino*iso*butyric acid by diaphragm is secondary to an increased rate of protein synthesis which draws amino acids in the medium into the tissue has been examined.

4. As the concentration of amino*iso*butyric acid is raised the effect of insulin on its accumulation by diaphragm disappears. Stimulation by insulin of incorporation of [^{14}C]glycine into protein is still observable with high concentrations of glycine.

5. In the presence of large concentrations of other amino acids the effect of insulin on the accumulation of amino*iso*butyric acid is markedly reduced; under conditions in which the rate of amino acid incorporation is inhibited by proflavin the effect of insulin on the accumulation of amino-*iso*butyric acid is again markedly reduced.

6. These results are consistent with, though they do not provide unequivocal support for, the hypothesis advanced for the means by which insulin stimulates accumulation of amino*iso*-butyric acid.

7. The possibility that insulin influences either the access of amino acids to the site of protein synthesis or the availability of energy-rich phosphate required for protein formation is discussed.

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Corticosteroid 21-Sulphates in Human Urine

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Pasqualini (1958, 1960) studied the distribution of the corticosteroids between the free, sulphate and glucuronide fractions in the urine of subjects after administration of adrenocorticotrophic hormone and established that an important fraction of corticosteroids was excreted in the urine as conjugates, contrary to the usual opinion (Neher, 1958; Peterson, Wyngaarden, Guerra, Brodie & Bunin, 1955; Peterson & Pierce, 1960). Corticosterone and 11-dehydrocorticosterone are found mainly in the ester sulphate fraction. The aim of the present research was to demonstrate the existence of corticosterone 21-sulphate and cortisol 21-sulphate in human urine.

EXPERIMENTAL AND RESULTS

Paper chromatography. Table 1 gives the R values of synthetic corticosteroid 21-sulphates relative to that of deoxycorticosterone 21-sulphate (Pasqualini, Zelnik & Jayle, 1960). For hydrolysis of the conjugates the developed dried chromatogram was suspended at 40° for 3·5 hr. in a covered cylindrical glass jar containing a mixture of conc. hydrochloric acid and dioxan (1:3, v/v) (Schneider & Lewbart, 1956).

Identification of corticosterone sulphate

Corticosterone was administrated orally to a normal subject in three 100 mg. doses at 8 hr. intervals. The urine was collected during the 48 hr. after the first dose. The

Table 1. *R values of corticosteroid 21-sulphates*

The solvents used were: system 1, toluene–*n*-butyl acetate–butanol–methanol–4N-NH_3 soln.–water (3:6:1:5:5, by vol.); system 2, toluene–*n*-butyl acetate–butanol–methanol–acetic acid–water (8:10:2:10:1:9, by vol.); system 3, propan-2-ol–hexane–NH_3 soln. (sp.gr. 0·925)–water (40:10:28:20, by vol.) (Cavina & Tentori, 1958). Whatman no. 1 filter paper and the descending technique were used. System 1 was run for 20 hr., system 2 for 40 hr. and system 3 for 16 hr. Temperature was 24±1°.

Compound	*R* relative to deoxycorticosterone 21-sulphate in system		
	1	2	3
Pyridinium deoxycorticosterone sulphate	1	1	1
Sodium deoxycorticosterone sulphate	1	1	1
Pyridinium corticosterone sulphate	0·40	0·58	0·70
Pyridinium cortisol sulphate	0·25	0·35	0·58

Table 2. *Urinary steroid output*

Steroid administered and determined	Steroid excreted (μg./l.)		
	Free	Ester sulphate	Glucuronide
Corticosterone	5	800	100
Cortisol	1200	700	120

urine, to which solid ammonium sulphate was added to give a 50% (w/v) solution, was extracted three times at pH 7 with an equal volume of ether–ethanol (3:1, v/v) (Edwards, Kellie & Wade, 1953). The extractions were made in separating funnels with continual shaking during 1 hr. The ethereal extracts were combined and dried. The dry urine extract was dissolved in water and the free corticosteroids were extracted by dichloromethane. The aqueous solution containing the conjugated corticosteroids was evaporated to dryness. These corticosteroids were separated on alumina into the sulphate and glucuronide fractions as described by Crepy, Jayle & Meslin (1957).

The ester sulphate fraction was purified by rechromatographing on alumina and, after elution, the ester sulphate fraction was further purified by dissolution in acetone–chloroform (3:1, v/v). This eliminated materials insoluble in this solvent mixture. The ester sulphate fraction of urine extracts was chromatographed first in solvent mixture 3 (Table 1). The zone that corresponded to the synthetic corticosterone sulphate used as a marker was eluted, and portions were chromatographed in solvent mixtures 1 and 2 (Table 1). The zone in solvent mixture 2 corresponding to the synthetic corticosterone 21-sulphate was eluted and hydrolysed by purified sulphatase obtained from *Helix pomatia* (Jarrige & Lafoscade, 1959). The liberated steroid was chromatographed with chloroform or benzene on paper treated with formamide, with toluene on paper treated with propanediol and, after acetylation, with *cyclo*hexane–benzene (1:1, v/v) on propanediol-treated paper (Burton, Zaffaroni & Keutman, 1951; Zaffaroni, 1953).

With the three solvents of Table 1 the zones on the chromatograms corresponding to corticosterone 21-sulphate gave positive methylene-blue reactions (Vlitos, 1953; Crepy & Judas, 1960); this is a colour reaction for ester sulphate. In the chromatogram with solvent mixture 1, the ester sulphate was identified by the rhodizonate test (Burma, 1953) and by the blue tetrazolium reaction, which was positive after hydrolysis in dioxan–hydrochloric acid mixture. Eluates from the zones showed $\lambda_{max.}$ 254 mμ. The free steroid obtained after hydrolysis with sulphatase had the same R_F as corticosterone in the three solvent systems and had $\lambda_{max.}$ 254 mμ. It gave a positive blue tetrazolium reaction and a yellow fluorescence in ultraviolet light. Its acetate behaved chromatographically as authentic corticosterone acetate.

Identification of cortisol sulphate

Cortisol was administrated orally to a normal subject in three 100 mg. doses at 8 hr. intervals. The urine was collected during the 36 hr. after the first dose. The urine, to which 5% (w/v) of sodium chloride had been added, was extracted three times with an equal volume of butanol at pH 7. The dried extract was dissolved in water and the free corticosteroids were extracted by dichloromethane. The conjugated corticosteroids were separated on alumina into sulphate and glucuronide fractions. The purification of ester sulphate fraction was the same as that used for corticosterone sulphate. The purified corticosteroid sulphate was hydrolysed with dioxan to which 3% (w/v) of trichloroacetic acid had been added (Cohen & Oneson, 1953).

Paper electrophoresis. Ethanol containing the ester sulphates was placed on strips of Carl Schleicher and Schüll, Dassel, no. 2043 (Zurich, Riedlistra 15. Switzerland) paper, in an apparatus of the Grassmann & Hannig (1952) type. The electrophoresis was at 60v for 7 hr. at 1 ma/cm. at 22° with 0·3N-ammonium carbonate buffer, pH 8·8.

Paper chromatography. The ester sulphates were successively fractionated in the three solvent systems of Table 1. A fraction of the eluate from the zone corresponding to the synthetic cortisol 21-sulphate, which had been chromatographed with solvent 1, was hydrolysed by sulphatase. Another part was hydrolysed with dioxan–trichloroacetic acid solution (Cohen & Oneson, 1953). The liberated steroid was chromatographed with chloroform on formamide-treated paper and in toluene–ethyl acetate–methanol–water (9:1:5:5, by vol.) (Bush, 1952), and its acetate with toluene on propanediol-treated paper. The free steroid and the acetate had the same migration characteristics as cortisol and cortisol acetate respectively. A further part of the extract was subjected to electrophoresis with synthetic cortisol sulphate as marker. The material was eluted and hydrolysed, and the liberated steroid was chromatographed with chloroform on formamide-treated paper. It had the same R_F as cortisol.

Table 2 gives the relative distribution of corticosterone and cortisol in the free, ester sulphate and glucuronide fractions.

SUMMARY

1. After administration of 300 mg. of corticosterone or cortisol to a man, the greater part of these two corticosteroids were found in the urine in ester sulphate fraction.

2. Evidence that sulphate was attached to C-21 was provided by the similarity of chromatographic behaviour with that of authentic corticosterone 21-sulphate or cortisol 21-sulphate, electrophoretic separation for cortisol sulphate and the reaction to tetrazolium being positive only after hydrolysis.

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The Biosynthesis and Metabolism of Polyols

SORBITOL (D-GLUCITOL) OF PLUM LEAVES

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Acylic polyhydric alcohols occur in many species of plants (Barker, 1955), although in the majority of cases neither quantitative data on the amounts present nor information on their metabolic role is available. Hypotheses have been advanced to explain the metabolism of polyols with little supporting evidence. Thus Donen (1939) suggested that sorbitol is formed in plum fruit when the concentration of hexoses reaches a critical level, whereas Kidd, West, Griffiths & Potter (1938) found that much of the sorbitol in pears is replaced by hexulose (probably fructose) as the fruit ripens. Sorbitol can also be utilized for polysaccharide synthesis in the leaves of Rosaceae and Gramineae (Barker, 1955; Altermatt & Neish, 1956), but nothing is known of the extent of this conversion under natural conditions.

Experiments with $^{14}CO_2$ originally designed to yield information on the biosynthesis of leaf polysaccharides (Andrews & Hough, 1958) indicated that sorbitol, the acyclic polyol in plum leaves, has an important role in the metabolism of these leaves. The incorporation of ^{14}C into sorbitol was considerably greater than into starch, and this observation initiated further studies of sorbitol metabolism in plum leaves.

Adcock (1957) assayed sorbitol by oxidation with sodium periodate and subsequent estimation of the two molecular equivalents of formaldehyde produced from the primary alcohol groups (O'Dea & Gibbons, 1953). Before oxidation, interfering substances were degraded by heating in sodium carbonate solution, and the degradation products removed by treatment with ion-exchange resins. However, when applied to plum-leaf extracts Adcock's method gave inconsistent results, a conclusion supported by White (1958), who suggested a preliminary acid hydrolysis of non-reducing carbohydrates such as sucrose to facilitate their degradation and removal. A simpler method

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for removing interfering substances was devised, in which the acid-hydrolysis step and ensuing alkaline degradation were effected in one process with ion-exchange resins. The modified assay procedure was used to estimate sorbitol in plum-leaf extracts.

A preliminary account of this work has been published (Anderson, Andrews & Hough, 1959).

MATERIALS AND METHODS

Experiments with $^{14}CO_2$

Radioactivity measurements. Compounds containing ^{14}C were burnt in a stream of O_2 in a micro-analytical combustion apparatus (F. J. McMurray, Dartford, Kent) and the $^{14}CO_2$ so produced was passed into hot $Ba(OH)_2$ solution (saturated at 20°). The precipitated $Ba^{14}CO_3$ was collected with suction on filter-paper circles (2·8 cm. diam.) as uniform disks (2 cm. diam.; wt. about 20 mg./cm.^{2}, equivalent to infinite thickness for counting), and washed with water, mounted on circular brass plates and dried at 120°.

Radioactivity was determined in a counter with a thin end-window Geiger tube (Twentieth Century Electronics Ltd., type EW. 3H); sufficient counts were taken to give a standard error of not more than ±3%. The counting equipment was calibrated in terms of $Ba^{14}CO_3$ by determining the count rate of 'infinitely thick' layers of $Ba^{14}CO_3$ prepared from poly([^{14}C]methyl methacrylate) of known specific activity, and a disk of this polymer was used as a standard reference source.

Labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks.

Incorporation of $^{14}CO_2$ *into plum leaves.* Spurs bearing four or five leaves, 7–10 cm. long, were removed from a plum tree (*Prunus domestica* var. Victoria) during a morning in early September, and the cut ends of the stems were immersed in water. After being kept in the dark for 24 hr. the spurs were exposed to light in the presence of $^{14}CO_2$. In each experiment about 20 leaves were used, sufficient to give about 2 g. of material soluble in aqueous methanol. The spurs were placed in a 4 l. desiccator, and $^{14}CO_2$ was generated *in situ* by adding lactic acid (80%, v/v, in water) to $Ba^{14}CO_3$ (about 1·8 g.; specific activity from 7·2 to 16·0 μc/m-mole in different experiments), sufficient to give an initial $^{14}CO_2$ concentration of 5%, v/v. The leaves received a steady illumination of about 400 ft.-candles and were allowed to incorporate and metabolize the $^{14}CO_2$ in the desiccator for periods of 1·5–8 hr. in different experiments. In experiments of longer duration the spurs were removed from the desiccator after the 8 hr. period and kept in a gentle current of air under approximately the same illumination, since trial experiments had shown that all the $^{14}CO_2$ supplied to the leaves was incorporated within 8 hr. For further details see Andrews & Hough (1958).

Isolation and characterization of [^{14}C]*sorbitol from plum leaves.* After photosynthesis the leaf laminae were quickly broken up, immediately dropped into boiling ethanol and extracted exhaustively with methanol in a Soxhlet apparatus. The combined ethanol and methanol extracts were submitted to paper chromatography, with butan-1-ol–ethanol–water (40:11:19, by vol.), butan-1-ol–pyridine–water (10:3:3, by vol.) or butan-2-one–acetic acid–saturated aq. boric acid (9:1:1, by vol.) (Rees & Reynolds, 1958) as mobile phase, and $AgNO_3$–NaOH (Trevelyan, Procter & Harrison, 1950) or *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) as spray reagent. Appropriate areas of the chromatograms were cut into 0·5 in. squares, which were examined for ^{14}C activity in the thin end-window counter.

[^{14}C]Sorbitol was isolated from alcoholic extracts as follows. After evaporation of the alcohol, the residues were suspended in water and treated with Bio-Deminrolit (The Permutit Co. Ltd., London, W. 4) mixed-bed ion-exchange resin (about 25 g./g. of dry alcohol extract) for 6–8 hr. at 90–100°. The neutral supernatant and aqueous washings of the resin were combined, concentrated to dryness and the residual syrups were dried over phosphoric oxide and weighed. Paper-chromatographic examination indicated that these syrups consisted mainly of sorbitol, so their weights were used in calculating the approximate amount of ^{14}C in sorbitol.

Portions of these syrups were submitted to chromatography on large sheets of Whatman no. 1 paper for 2 days, with butan-1-ol–pyridine–water (10:3:3, by vol.) as mobile phase. Marker strips were then sprayed with the $AgNO_3$–NaOH reagent to detect sorbitol, and the appropriate regions of the unsprayed paper were cut out and eluted with water. The eluates were evaporated to dryness and the residues acetylated by treatment overnight at room temperature with excess of acetic anhydride in a large excess of anhydrous pyridine (Verley & Bölsing, 1901). Water and chloroform were added, then the organic phase was separated, washed successively with 2N-HCl, saturated $NaHCO_3$ solution and water, dried with $CaCl_2$, decolorized with charcoal and evaporated. The reaction products crystallized, and recrystallization from ethanol gave [^{14}C]sorbitol hexa-acetate with m.p. 98–99° in each case, undepressed on admixture with an authentic sample. This derivative was used to determine the specific activity of [^{14}C]sorbitol from the photosynthesis experiments; further recrystallizations produced no change in specific activity.

[^{14}C]*Sorbitol from its hexa-acetate.* Crystalline [^{14}C]-sorbitol hexa-acetate (250 mg.) was dissolved in dry chloroform (25 ml.) and a solution of sodium methoxide in methanol (5%, w/v; 3 ml.) added. After 2 hr. at 20°, the precipitated [^{14}C]sorbitol was centrifuged off and dissolved in water, then the solution was treated with Amberlite IR-120(H) resin to remove traces of Na^+ ions and evaporated to yield [^{14}C]sorbitol as a colourless glassy solid.

Isotopic-dilution analysis for sorbitol in a plum-leaf extract. [^{14}C]Sorbitol (103·4 mg.), which on combustion had given $Ba^{14}CO_3$ with a specific activity of 499 counts/min. at infinite thickness under standard conditions, was dissolved in a portion (250 ml.) of an alcoholic extract of non-radioactive plum leaves (≡3·75 g. dry wt.). After evaporation of the solvent, crystalline sorbitol hexa-acetate was prepared from the residue. After recrystallization this derivative gave $Ba^{14}CO_3$ with a specific activity of 62·7 counts/min. at infinite thickness under the same conditions as before.

Isolation of [^{14}C]*glucose from plum-leaf starch.* The alcohol-insoluble leaf residues from each photosynthesis experiment were dried, weighed, assayed for ^{14}C and then extracted successively with hot water, cold N-NaOH and hot 2·5N-NaOH. The three fractions so obtained from each batch of leaves were hydrolysed separately (N-H_2SO_4; 8 hr. at 100°) and the resultant monosaccharides were

separated by paper chromatography with butan-1-ol–ethanol–water (10:3:3, by vol.) as mobile phase. Glucose was eluted with water from the appropriate sections of the chromatograms, then the eluates were evaporated under reduced pressure, the residues were redissolved in water and the resultant solutions were passed through columns of Amberlite IR-120(H) and IRA-400(CO_3) resins and finally evaporated to dryness. The resultant syrups, after paper-chromatographic examination as a check on purity, were dried over phosphoric oxide and weighed, then inactive D-glucose was added and the sugars were crystallized and assayed for ^{14}C activity.

Quantitative estimation of sorbitol

Sodium metaperiodate reagent. The grade described as 'for glycerol determinations' (Hopkin and Williams Ltd.) was used. A 0·015M solution was prepared as required by diluting a stock 0·3M solution, which was stored in the dark in a stoppered flask. All manipulations involving periodate solutions were carried out in as dim a light as was consistent with accuracy.

Sodium periodate–sodium hydrogen carbonate solution. Equal volumes of 0·015M-sodium metaperiodate and a freshly prepared saturated (at 20°) solution of $NaHCO_3$ were mixed immediately before use.

Lead dithionate solution. Barium dithionate was converted into the lead salt by the method of O'Dea & Gibbons (1953), giving an aq. 15% (w/v) solution, which was stored at 0°.

Chromotropic acid reagent. Chromotropic acid specially prepared 'for formaldehyde determinations' (Hopkin and Williams Ltd.) was dissolved in H_2SO_4 as described by O'Dea & Gibbons (1953) and the solution was stored in the dark.

Periodate method for sorbitol estimation. The periodate-oxidation reaction was performed in tapered 10 ml. centrifuge tubes, by adding sodium periodate–$NaHCO_3$ soln. (1 ml.) to the solution (2 ml.) for assay, containing not more than 200μg. of sorbitol. After 1 hr., excess of periodate was destroyed by adding lead dithionate solution (1 ml.), then the precipitated lead salts were moved by centrifuging and a portion (1 ml.) of the clear supernatant was added to chromotropic acid reagent (10 ml.) contained in a 50 ml. centrifuge tube. Precipitated lead sulphate was removed on the centrifuge and the clear supernatant was heated in a stoppered tube in a darkened, vigorously boiling-water bath for exactly 30 min. Thereupon the tube was cooled to room temperature in running tap water and the light-absorption of the solution measured in an EEL colorimeter with a no. 626 filter. Standard curves were prepared for the range 15–200μg. of sorbitol in 2 ml. of solution. Although deviations from linearity were noted in the upper part of the calibration curve, where the periodate concentration becomes limiting, reproducible results were obtained with an accuracy of $\pm 3\%$.

Estimation of sorbitol in the presence of sucrose. Sorbitol (0·4–2·2 g.) and sucrose (about 0·5 g.) were dissolved in water (50 ml.) and the solutions were diluted to 1 l. with ethanol. Portions (10 ml.) were transferred to 50 ml. conical flasks and the solvents evaporated on a boiling-water bath. Amberlite IR-120(H) (3 g.) and IR-400(OH) (10 g.) resins were added with sufficient water to cover them, and heating was continued with addition of more water as necessary. After 6 hr. the contents of the flasks, including the resins, were washed into glass columns (1 cm. × 35 cm.), each containing about 10 ml. of a mixture of equal parts of Amberlite IR-120(H) and IR-400(OH) resins. Complete elution of the sorbitol was effected by passing water (230 ml.) through the columns. The effluents were collected in graduated flasks, diluted to 250 ml. with water and portions (2 ml.) taken for estimation of sorbitol by the periodate method as described above.

Estimation of sorbitol added to plum-leaf extracts. Sorbitol (10–100 mg.) was added to portions (50 ml., containing roughly 30 mg. of sorbitol) of the alcoholic extracts of two batches of plum leaves. The alcohol was evaporated, the residues and added sorbitol were dissolved in water and suitable portions used for estimation of sorbitol as described above for sorbitol–sucrose mixtures.

Sorbitol content of plum leaves during 20 hr. Three plum trees (var. Victoria) in an orchard at Long Ashton, Somerset, were selected for this experiment. During warm, dry weather in July, five leaves were removed randomly from each tree at the intervals of time shown in Table 5. The leaf laminae were broken up and plunged into boiling ethanol (50 ml.) immediately after picking. The insoluble material was filtered off and extracted with methanol in a Soxhlet apparatus for 2 days. The final alcohol-insoluble residues were dried, at first at 60° and then over phosphoric oxide *in vacuo*, and weighed. The combined extracts from each group of 15 leaves were diluted to 500 ml. Samples (10 ml.) were evaporated, the residues treated with ion-exchange resins as described above, and the final solutions were used for sorbitol estimations by the periodate method.

RESULTS AND DISCUSSION

An investigation of polysaccharide metabolism in plum leaves with $^{14}CO_2$ (Andrews & Hough, 1958) was extended by studying, at the same time, the incorporation of ^{14}C into the alcohol-soluble fraction of the leaves. Mature Victoria-plum leaves, which had been kept in the dark for 24 hr. to deplete their starch content, were allowed to photosynthesize in an atmosphere containing initially 5% (v/v) of $^{14}CO_2$; then they were extracted exhaustively with methanol, and the extracts were submitted to paper chromatography. When the chromatograms were monitored for ^{14}C activity, a major part of the activity was located in the main polyol constituent of the leaves, after periods of photosynthesis from 1·5 to 24 hr.

This polyol was isolated and characterized as sorbitol (D-glucitol). No other hexitol or acyclic polyol was found in plum-leaf extracts by paper chromatography in three solvents capable of distinguishing sorbitol from other polyols, although a trace of a compound with a rate of movement on the chromatogram less than that of sorbitol, and corresponding to *myo*inositol, was detected. Further evidence that little, if any, acyclic polyol other than sorbitol is present in plum leaves in the free state was obtained by the agreement between an estimate of the concentration of acyclic polyol in a non-radioactive plum-leaf

extract by the adapted periodate method (Found: 0·67 g./l.) and an isotopic-dilution assay for sorbitol in the same extract (Found: 0·68 g./l.), with [^{14}C]sorbitol purified via its crystalline hexa-acetate as carrier.

As shown in Table 1, the yield of crude sorbitol from the leaves of each photosynthesis experiment was approximately the same, relative to the quantity of leaves used, and corresponded to 38–45 mg./g. dry wt. of leaf. No systematic variation in these values was apparent, but judging from the weights of D-glucose isolated from hydrolysates of the polysaccharide fractions of the same leaves, considerable net synthesis of starch occurred during the same period.

The quantity of impure sorbitol isolated from the leaves, before chromatographic purification, was considered a good measure of the amount initially present, but experience indicated that the losses incurred in isolating and purifying the glucose were 40–60%, depending on the amount involved. Therefore in calculating the approximate incorporation of ^{14}C into sorbitol and starch, allowance was made for a 50% loss of glucose in the preparation, and the resulting uncertainty in the glucose figure is indicated in Table 2, where the results are depicted together with other figures relating to ^{14}C distribution in the leaves. After photosynthesis for 6 hr. practically all of the $^{14}CO_2$ supplied to the leaves had been metabolized (Andrews & Hough, 1958) and, in these circumstances, since the same amount of carbon dioxide was supplied each time, the resultant specific activities of the leaf components would be related inversely to the weights of the leaves used. Accordingly the specific activities of sorbitol at 6, 15·5 and 24 hr. were corrected to a common basis of 3 g. of alcohol-insoluble leaf residue per sample (actual weights were 3·12, 3·72 and 2·97 g. respectively). In every experiment a large proportion of the ^{14}C in alcohol-soluble compounds was present in the sorbitol, demonstrating a rapid and high degree of incorporation of isotope into this polyol. In each case there was also considerably more ^{14}C in sorbitol than in starch. The results in Table 1 indicate that no net synthesis of sorbitol occurred during photosynthesis, yet the specific activity of ^{14}C in sorbitol relative to that of the $^{14}CO_2$ supplied (Table 2) shows that, after photosynthesis for only 3 hr. in a high $^{14}CO_2$ concentration, 15% of the sorbitol was newly synthesized from labelled precursors. This corresponds to the synthesis and utilization of about 2·0 mg. of sorbitol by each leaf. Clearly there was a rapid turnover of the sorbitol pool in equilibrium with the primary products of photosynthesis. The specific activity of ^{14}C in starch was different in each polysaccharide fraction examined (P. Andrews & L. Hough, unpublished work), but usually about twice that of ^{14}C in sorbitol. After photosynthesis for 3 hr. roughly 0·7 mg. of starch had been synthesized per leaf from labelled precursors.

The apparent invariance of sorbitol concentration in plum leaves under the rather extreme conditions of the photosynthesis experiments was unexpected, but seemed worthy of further investigation, particularly with leaves still growing on the tree. The method for estimating sorbitol in bio-

Table 1. *Glucose and sorbitol isolated from photosynthesizing plum leaves*

For experimental details see text.

Period of photosynthesis (hr.)	Yield corresponding to 3 g. of alcohol-insoluble residue*	
	Sorbitol (mg.)	D-Glucose† (mg.)
1·5	275	‡
3	330	18
6	270	25
15·5	310	29
24	280	53

* In separate experiments the actual weight of alcohol-insoluble residue was in the range 2·5–3·7 g.
† From polysaccharide hydrolysates.
‡ Insufficient glucose was present to permit its isolation and purification.

Table 2. *Distribution of ^{14}C in plum-leaf constituents after photosynthesis in $^{14}CO_2$*

See text for experimental details.

Period of photosynthesis (hr.)		1·5	3	6	15·5	24
As % of ^{14}C supplied	Total ^{14}C in leaves	34	79	98	97	91
	^{14}C in alcohol-soluble fraction	25	57	59	53	52
	^{14}C in sorbitol	7	17	21	30	22
	^{14}C in starch	—	5±2	8±2	11±2	17±3
As % of total ^{14}C in leaves	^{14}C in alcohol-soluble fraction	72	68	59	58	57
	^{14}C in sorbitol	20	22	22	31	25
	^{14}C in starch	—	6±2	8±2	11±2	19±3
	^{14}C in sorbitol (as % of ^{14}C in alcohol-soluble materials)	28	32	39	53	43
$\frac{\text{Specific activity of } ^{14}\text{C in sorbitol}}{\text{Specific activity of } ^{14}CO_2 \text{ supplied}}$		0·08	0·16	0·23	0·26	0·22

logical materials described by Adcock (1957) gave inconsistent results with plum-leaf extracts. The improvement suggested by White (1958) to remove interfering non-reducing carbohydrates may well have been effective here, but a simplified method with ion-exchange resins to hydrolyse, degrade and remove interfering substances was devised instead.

Sucrose is an alcohol-soluble constituent of plum leaves, and White (1958) showed that it interferes with Adcock's method of sorbitol estimation. However, the simplified method gave satisfactory results with mixtures of sorbitol and sucrose, initially dissolved in 95% ethanol to simulate leaf extracts and containing 0·6–4 times the concentration of sorbitol found in plum-leaf extracts (Table 3). To test for possible diurnal variations in substances interfering with estimations by the periodate method, the sorbitol concentrations in extracts of plum leaves picked at 3 a.m. and 8 p.m. were determined before and after the addition of known amounts of sorbitol. The results (Table 4) again were satisfactory.

Table 3. *Estimation of sorbitol admixed with sucrose*

Figures are concentrations in g./l.

Composition of mixture		Sorbitol found
Sucrose	Sorbitol	
0·484	0·400	0·416
0·646	0·788	0·780
0·568	2·232	2·280

Table 4. *Estimation of sorbitol added to plum-leaf extracts*

Figures are concentrations in g./l.

Sorbitol added	Estimated increment in sorbitol
(*A*) With an extract of leaves picked at 3 a.m. Initial sorbitol concentration: 0·566 g./l.	
0·368	0·372
0·734	0·730
1·218	1·140
2·042	1·815
(*B*) With an extract of leaves picked at 8 p.m. Initial sorbitol concentration: 0·604 g./l.	
0·276	0·278
1·804	1·750

Table 5. *Sorbitol content of plum leaves during 21 hr. under natural conditions*

Fifteen leaves were picked at each time, giving 2–3 g. of alcohol-insoluble leaf residue.

Time of sampling	Sorbitol in alcohol extract (mg./g. of alcohol-insoluble leaf residue)
1 p.m.	106
5 p.m.	112
8 p.m.	111
Midnight	112
3 a.m.	110
5 a.m.	105
9 a.m.	109

The method was then applied to the estimation of sorbitol in extracts of plum leaves picked from three trees at intervals during a 20 hr. period, commencing at 1 p.m. one day and ending at 9 a.m. the next day. The results (Table 5) for each sample do not differ significantly from their mean value of about 110 mg. of sorbitol/g. of alcohol-insoluble leaf residue (corresponding to about 45 mg./g. dry wt. of leaf), although the leaves were in darkness from about 10 p.m. to 4 a.m. Paper-chromatographic examination of the extracts indicated that sorbitol was the only polyol constituent, except for traces of *myo*inositol, which does not give formaldehyde on periodate oxidation. The evidence from this experiment and from the photosynthesis experiments with $^{14}CO_2$ indicates that the total free sorbitol content of plum leaves is independent of the photosynthetic activity of the leaves, both under natural conditions and during a period of very rapid sorbitol synthesis after a prolonged spell in darkness.

These results may be compared with findings of Hutchinson, Taper & Towers (1959), who observed a high degree of incorporation of $^{14}CO_2$ into the sorbitol of apple leaves and suggested that the polyol has a central role in metabolism of the leaves. A large incorporation of $^{14}CO_2$ into the D-mannitol of *Fucus vesiculosus* has likewise been reported (Bidwell, Cragie & Krotkov, 1958). A metabolic function of a polyol which has been conclusively demonstrated is the interconversion of *threo*-D- and -L-pentulose (xylulose) via xylitol in guinea-pig liver (Hollman & Touster, 1957), and so it seems possible by analogy that sorbitol is an intermediate in the interconversion of D-glucose, D-fructose and L-sorbose.

SUMMARY

1. Plum leaves, which are rich in sorbitol, utilized a higher proportion of the ^{14}C incorporated during photosynthesis in $^{14}CO_2$ for sorbitol synthesis than for starch synthesis.

2. Despite the large amounts present in the leaves, sorbitol is rapidly equilibrated with the primary products of photosynthesis; 15% was newly synthesized from ^{14}C-labelled precursors in 3 hr.

3. The method of Adcock (1957) for estimating sorbitol in biological material was adapted for use with leaf extracts.

4. The sorbitol content of plum leaves under natural conditions was followed from 1 p.m. on one day to 9 a.m. the next day. The amount present remained constant at 110 mg./g. of alcohol-insoluble leaf residue (45 mg./g. dry wt. of leaf).

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A New Asparagine Derivative, N^4-(2-Hydroxyethyl)-L-asparagine, from Bryony (*Bryonia dioica*)

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Many amino acids additional to those normally present in protein have been identified as components of higher plants (see reviews, Hunt, 1959; Fowden, 1958, 1960). Several of the newly characterized acids are derivatives of either glutamic acid or glutamine; others based on aspartic acid or asparagine are now known. N^4-Ethyl-L-asparagine (I) was isolated recently from squirting cucumber (*Ecballium elaterium*) by Gray & Fowden (1961). The present paper reports the isolation of larger quantities from another member of the Curcurbitaceae, *Bryonia dioica* (white bryony), together with another substituted amide identified as N^4-(2-*hydroxyethyl*)-L-*asparagine* (II).

$$HO_2C\cdot CH(NH_2)\cdot CH_2\cdot CO\cdot NH\cdot C_2H_5 \quad (I)$$

$$HO_2C\cdot CH(NH_2)\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot OH \quad (II)$$

ISOLATION AND IDENTIFICATION

The substances were encountered as ninhydrin-reacting spots (brown) on paper chromatograms of 75% (v/v) ethanol extracts of macerated bryony shoots. About 7 g. of ethylasparagine and 1·2 g. of hydroxyethylasparagine were isolated from an aqueous extract of 20 kg. of shoots. Hydroxyethylasparagine, having empirical formula $C_6H_{12}O_4N_2$, gave equimolar amounts of aspartic acid and ethanolamine after acid hydrolysis. Extracts of rat liver also converted hydroxyethylasparagine into aspartic acid and ethanolamine; ethylasparagine was converted similarly into aspartic acid and ethylamine. The pK_A values of hydroxyethylasparagine were 2·32 and 8·92 [cf. values for asparagine, 2·02 and 8·80 (Meister, 1957)]. Ammonia displaced hydroxyethylasparagine from cation-exchange resin columns together with glutamic acid and before displacement of ethylasparagine and asparagine. This result was in agreement with structure for hydroxyethylasparagine which contains a free hydroxyl group; a possible alternative structure, 4-(2-aminoethyl)-hydrogen aspartate, an ester of ethanolamine and aspartic acid, presumably would behave as a basic amino acid on ion-exchange resins.

Confirmation of the hydroxyethylasparagine structure was obtained by chemical synthesis. The

synthetic and natural compounds were shown to be identical by comparison of their m.p., $[\alpha]_D^{20}$, R_F values and infrared spectra. The spectra confirmed the absence of an ester linkage.

EXPERIMENTAL

Chromatographic techniques. Paper chromatograms were run on Whatman no. 3MM chromatographic-grade filter paper. Sheets were washed thoroughly with 5% (v/v) acetic acid solution before being used for preparative work. Solvents used included: *A*, 75% (w/v) phenol solution in the presence of NH_3; *B*, a one-phase mixture of butan-1-ol–acetic acid–water (90:10:29, by vol.); *C*, 70% (v/v) propan-1-ol; *D*, the upper phase of ethyl acetate–pyridine–water (2:1:2, by vol.). A 0·1% (w/v) solution of ninhydrin in 95% (v/v) ethanol was normally used as a spraying agent.

Detection of ethyl- and hydroxyethyl-asparagine in bryony shoots. Two-dimensional paper chromatograms (solvent *A* followed by solvent *B*) of 75% (v/v) ethanol extracts of macerated bryony leaves and stems showed three strong brown spots after treatment with ninhydrin. Two of these were identified as asparagine and *N*-ethylasparagine (I); the latter partially overlapped the yellow spot of proline. The third brown spot (hydroxyethylasparagine; R_F in solvent *A*, 0·65) partially overlapped glutamine (R_F 0·61); the two substances had identical R_F values in solvent *B*. Ethyl- and hydroxyethyl-asparagine were not detected on chromatograms prepared from seed extracts.

Preliminary fractionation on cation-exchange resins. Bryony shoots (20 kg.) were collected in August and kept at −40°. The frozen material was crushed and batches (250 g.) were macerated with chloroform-saturated water (500 ml.) at 2°. The macerate was pressed (6000 lb./in.^{2}); the expressed juice was adjusted immediately to pH 4·6–4·8 and warmed (60°) for 10 min. to flocculate the protein.The combined filtrate (about 50 l.) was applied at a rate of 2 l./hr. to a Zeo-Karb 215 (resin mesh 80–100) column (120 cm. × 10 cm. diam.).

All the amino acids were adsorbed. The column was washed with 200 l. of water and eluted with 0·5N-NH_3 solution. Amino acids were present in 12 l. of eluate collected in 500 ml. fractions. Resolution was not good, but the first five fractions contained only aspartic acid, glutamic acid, threonine and serine, and were discarded. All later fractions were combined and evaporated *in vacuo* at 50° to remove NH_3. The sticky residue was redissolved in water (4 l.), and the solution (pH 6·8) was decolorized by treating it three times with charcoal (50 g., prepared as by Partridge, 1949).

This solution was applied at the rate of 50 ml./hr. to Dowex 50 X 8 (mesh 100–200) columns (upper: 60 cm. × 3·5 cm. diam.; lower: 30 cm. × 2 cm. diam.). After adsorption the amino acids formed a band of depth 27 cm. upon the upper column. The columns were washed with water (6 l.) and then were eluted with 0·25N-NH_3 solution (flow rate 50 ml./hr.). After the break-through of aspartic acid 85 fractions (15 ml.) were collected. Hydroxyethylasparagine was present in fraction nos. 7–16 together with aspartic acid, glutamic acid, an unidentified amino acid (purple colour with ninhydrin, designated P1) and small amounts of threonine and serine. Ethylasparagine was present in fraction nos. 15–38 and asparagine (in small amount) in nos. 23–36.

Isolation of N^4*-ethyl-*L*-asparagine* (*I*). Fraction nos. 18–33 contained large amounts of ethylasparagine (about 10 g.) together with traces of several other amino acids. The combined fractions were evaporated *in vacuo* and the residue was recrystallized twice from 75% (v/v) ethanol to yield pure ethylasparagine (7·15 g.). The properties and synthesis of ethylasparagine have been reported by Gray & Fowden (1961).

Isolation of N^4*-(2-hydroxyethyl-*L*-asparagine* (*II*). On storage at 2° aspartic acid (1·8 g.) separated from fraction nos. 5–9 and glutamic acid (1·1 g.) from nos. 12–16. After removal of the crystals, fraction nos. 7–14 were pooled and evaporated *in vacuo* (yield 3·8 g.). This residue was redissolved in water (250 ml.) and the solution was applied to a Dowex 1 (acetate form; resin mesh 100–200) column (30 cm. × 2 cm. diam.) to absorb residual aspartic acid and glutamic acid. The column was washed with water (500 ml.) and, after evaporation of the combined eluates, the residue (1·9 g.) contained hydroxyethylasparagine and P1 together with traces of threonine and serine. The residue, dissolved in a minimum volume of warm water, was streaked across eight sheets of washed filter paper (27 in. × 23 in.), which were developed with solvent *A*. The four amino acids were resolved completely and the bands of hydroxyethylasparagine and P1 were cut out and eluted with water. After clarification with charcoal and evaporation, hydroxyethylasparagine (1·22 g.) and P1 (0·3 g.) were obtained. Recrystallization from 80% (v/v) ethanol gave pure hydroxyethylasparagine as lustrous plates (760 mg.).

Properties of hydroxyethylasparagine. This had m.p. 199–200° and $[\alpha]_D^{20}$ −2·9±0·5° in water (*c*, 5·0) (Found: C, 40·6, 40·4; H, 7·1, 6·9; N, 15·8, 15·9. $C_6H_{12}O_4N_2$ requires C, 40·9; H, 6·9; N, 15·9%). When a 0·1M-solution of hydroxyethylasparagine was titrated against 0·1N-HCl, a pK_A of 2·32 was determined; titration against 0·1N-NaOH gave a pK_A of 8·92.

Hydroxyethylasparagine was destroyed completely when heated with 6N-HCl at 100° for 24 hr. Paper chromatography showed two products, which were inseparable from aspartic acid and ethanolamine in solvents *A*–*D*. Quantitative paper chromatography (Fowden & Webb, 1958; Bryant & Fowden, 1959) with solvent *B* established that 17·6 mg. of hydroxyethylasparagine (100 μmoles) gave 13·0 mg. of aspartic acid (98 μmoles) and 6·3 mg. of ethanolamine (103 μmoles).

Under the conditions used for quantitative conversion of asparagine into NH_3 and aspartic acid, namely 2N-HCl at 100° for 3 hr., less than 50% of hydroxyethylasparagine was hydrolysed. The hydrolysate contained an intermediate compound, giving a yellow ninhydrin-reacting spot of $R_F \sim 0{\cdot}95$ in solvent *A*. This intermediate, possibly a cyclization product, disappeared during prolonged hydrolysis with 2N-HCl.

Enzymic degradation. Fresh rat liver was homogenized with 2 vol. of phosphate buffer (pH 7·8) containing 100 ml. of 0·2M-KH_2PO_4 and 90·4 ml. of 0·2N-KOH/l. After centrifuging at 10 000*g* for 15 min., the supernatant was concentrated to one-third of the original volume by dialysis against moistened Carbowax 20M polyethylene glycol (supplied by G. T. Gurr Ltd., London, S.W. 6). The concentrate was dialysed against several changes of 0·1M-phosphate (pH 7·8) for 24 hr. at 2° and then used as a source of crude asparaginase (Greenstein & Carter, 1946).

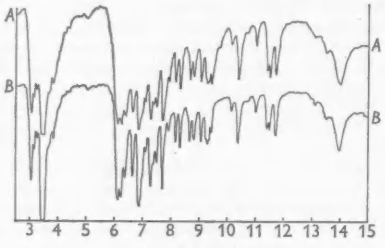

Fig. 1. Infrared spectra of N^4-(2-hydroxyethyl)-L-asparagine. *A*, Synthetic material; *B*, natural material. Both spectra were determined with liquid-paraffin mulls of the amide.

The hydrolysis of asparagine and ethyl- and hydroxyethyl-asparagine by the liver preparation was studied at 37°. After 3 hr., ethylamine and aspartic acid were identified chromatographically as degradation products of ethylasparagine, whereas hydroxyethylasparagine gave ethanolamine and aspartic acid. Asparagine was hydrolysed faster than either ethyl- or hydroxyethyl-asparagine.

The rate of breakdown of asparagine by rat-liver extracts was increased markedly when a keto acid, particularly pyruvic acid, was added to the incubation mixtures (Greenstein & Carter, 1946; Meister, Sober, Tice & Fraser, 1952). Under these conditions the α-amino group of asparagine is donated to the added keto acid; the amide group is also hydrolysed and oxaloacetate and NH_3 form the main reaction products. The rate of disappearance of either ethyl- or hydroxyethyl-asparagine from reaction mixtures was not influenced appreciably by the addition of pyruvate and so transamination of the substituted amides must have been negligible. This behaviour was similar to that of L-*iso*asparagine (Meister *et al.* 1952).

Synthesis of N^4*-(2-hydroxyethyl)-*L*-asparagine (II).* 4-Ethyl hydrogen L-aspartate was prepared by the method of Curtis & Koch (1888). Dry HCl gas was bubbled through a suspension of L-aspartic acid (20 g.) in dry ethanol (200 ml.) until no solid remained. The solution was evaporated to dryness *in vacuo* at 40°. The residue was dissolved in ethanol and re-evaporated to remove traces of HCl when 4-ethyl hydrogen L-aspartate hydrochloride (25 g.) remained. An equivalent amount of methanolic KOH was added to 4-ethyl hydrogen L-aspartate hydrochloride (20 g.) dissolved in ethanol. After removal of precipitated KCl, the filtrate was concentrated and 4-ethyl hydrogen L-aspartate (12·5 g.) crystallized.

N^4-(2-Hydroxyethyl)-L-asparagine was prepared by refluxing 4-ethyl hydrogen L-aspartate (8 g.) for 3 hr. with 10 ml. of ethanolamine dissolved in 10 ml. of ethanol. Ethanol was removed by evaporation *in vacuo*, and the sticky residue was dissolved in water (300 ml.). This solution was fractionated on a Dowex 50X 8 (mesh 100–200) column (30 cm. × 2 cm. diam.). During elution with 0·2N-NH_3 solution, hydroxyethylasparagine and some aspartic acid appeared together in the early fractions; unchanged ethanolamine and small quantities of ninhydrin-reacting by-products appeared in later fractions. Hydroxyethylasparagine was freed from aspartic acid by absorbing the latter on a Dowex 1 (mesh 100–200) column (20 cm. long, 1·5 cm. diam.). Evaporation of the eluate left a residue of hydroxyethylasparagine (5·1 g.). Recrystallization from 80% (v/v) ethanol (50 ml.) gave pure hydroxyethylasparagine (3·2 g.), m.p. 202–203°, $[\alpha]_D^{20}$ $-2·5 \pm 0·4°$ in water (*c*, 5·0) (Found: C, 40·3; H, 6·8; N, 15·9. $C_6H_{12}O_4N_2$ requires C, 40·9; H, 6·9; N, 15·9%).

The synthetic material was hydrolysed completely by treatment with 6N-HCl at 100° for 24 hr. to yield aspartic acid and ethanolamine.

Identity of natural and synthetic amides. The two substances showed identical chromatographic behaviour in all four chromatographic solvents. The agreement between their m.p. and $[\alpha]_D^{20}$ values and their behaviour upon treatment with HCl solutions at 100° also served to confirm their identity. Final proof of identity was obtained by comparison of their i.r. spectra, which showed that all absorption peaks were common to both substances (Fig. 1). No absorption peaks were observed at wavelengths (5·6–5·7 μ) characteristically associated with ester groups.

SUMMARY

1. N^4-(2-Hydroxyethyl)-L-asparagine has been newly characterized as a component of higher plants, being isolated from shoots of *Bryonia dioica* (white bryony) together with larger amounts of N^4-ethyl-L-asparagine.

2. N^4-(2-Hydroxyethyl)-L-asparagine was converted into an equimolar mixture of aspartic acid and ethanolamine on hydrolysis with hydrochloric acid. Hydrolysis proceeded more slowly than that of the parent amide, asparagine.

3. N^4-(2-Hydroxyethyl)-L-asparagine was prepared by refluxing an ethanolic solution of 4-ethyl hydrogen L-aspartate with ethanolamine.

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The Metabolism of Sulphatides in Cerebral Tissues

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The sulphatides of brain consist essentially of galactocerebrosides, the galactose moiety of which is singly esterified on C-6 by sulphuric acid (Thannhauser, Fellig & Schmidt, 1955). The various preparations described by Blix (1933), Lees, Folch, Sloane-Stanley & Carr (1959), Thannhauser *et al.* (1955) and Jakewitz (1960*b*), if they differ, would appear to do so only in the nature of the fatty acid part of the parent cerebrosides (Lees *et al.* 1959; Jakewitz, 1960*b*).

The metabolism of brain sulphatides has been little studied. In a classical investigation it was shown that the sulpholipids of rat brain increased in quantity throughout life (Koch & Koch, 1913), a study which has not since been repeated. More recently, marked increases in the quantities of sulpholipids in human brain have been demonstrated in certain demyelinating diseases (Austin, 1959; Jakewitz, 1960*a*, *b*).

Experiments with radioactive sulphate *in vivo*, however, led to the conclusion that in normal brain the sulphatides are relatively metabolically inert. Thus, after the injection of radioactive sulphate into rats or rabbits, the radioactivity accumulating in the brain sulpholipids was extremely low and declined very slowly over periods of up to several weeks (Bostrom & Odeblad, 1953; Dziewiatkowski, 1953; Radin, Martin & Brown, 1957; Green & Robinson, 1960). Such experiments, however, parallel in their results earlier experiments of a similar nature conducted with radioactive phosphate [see Heald (1960) for review], in which the low radioactivity of the phospholipids was wrongly attributed to a generally slow metabolism rather than to a slow rate of penetration of radioactive phosphate into the brain. It was therefore considered desirable to re-examine the incorporation of sulphate into the sulpholipids of brain under conditions *in vitro* where more precise information might be obtained. Since it has been shown (Bostrom & Odeblad, 1953) that the cortical surfaces of rat brain take up sulphate more rapidly than other areas, the work was performed on slices of guinea-pig cerebral cortex.

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MATERIALS AND METHODS

Tissues and salines. Slices of adult-guinea-pig cerebral cortex were used. Animals were killed by a blow on the neck and rapidly exsanguinated. Three slices, 0·30–0·35 mm. thick, were cut from each hemisphere by using a recessed plate as a guide and a strip of razor blade (McIlwain, 1951). Each slice was floated into the appropriate saline and trimmed rapidly with a scalpel. Excess of saline was removed by draining against a glass plate. The slices were weighed on a torsion balance, and adjusted to a weight of 110–120 mg. by trimming with scissors if necessary to standardize as far as possible the tissue–fluid ratios in each vessel. They were again floated into saline and transferred to manometric vessels. Since two slices of cortex were required for a single estimation of sulphatide, experiments were arranged so that, with the six slices obtained from a single brain, two different treatments were compared with a control either containing glucose or without any added substrate. Each vessel contained 3·5 ml. of saline composed of: NaCl, 127 mM; KCl, 6·3 mM; $MgCl_2$, 1·28 mM; tris, 0·05 M (adjusted to pH 7·4 with HCl). Slices were incubated at 37·5° under O_2. Substrates in the concentrations stated in the appropriate Tables were included in the salines in which the slices were cut and trimmed. 2:4-Dinitrophenol was added from the side arms of the manometric vessels.

Determination of sulphatides

These were determined as sulphate released on hydrolysis (Folch & Lees, 1959) essentially by the method of Long & Staples (1961) (cf. Jones & Leytham, 1956), modified to permit the determination of radioactive sulphate in extracts of tissue slices.

Preparation of extracts. After incubation each pair of slices was tipped out into a basin and the slices were rapidly drained and transferred to 5 ml. of chloroform–methanol (2:1, v/v). The incubation media were retained for the determination of radioactivity in appropriate samples. The slices were dispersed in the chloroform–methanol by grinding in a small centrifuge tube with a fitting pestle for 3–4 min. and centrifuged at 12 000*g* for 10 min. at −5°. The supernatants were removed, the residues again extracted with a further 5 ml. of chloroform–methanol, and the combined supernatants from each pair of slices were brought to 10 ml. with chloroform–methanol. To each extract 0·05 M-NaCl (0·2 vol.) was added, and after vigorous shaking the mixtures were centrifuged and the upper layers removed and discarded. The lower layers were again extracted three times with 2 ml. of chloroform–methanol–0·05 M-NaCl (5·2:50:47·4, by vol.),

and the upper layers discarded. This procedure, designed for the removal of non-lipid substances from lipid extracts (Folch, Lees & Sloane-Stanley, 1957), completely removed all traces of radioactive sulphate added to the extracts.

Determination of sulphatide sulphur. The washed lipid extracts were evaporated to small volume and made up precisely to 1·0 ml. with ethanol or chloroform–methanol. A portion (0·8 ml.) of the extract was transferred to conical tubes (3 ml. capacity) and evaporated to dryness in a stream of N_2. To the residues HCl (5·5 N; 0·3 ml.) was added, and the tubes were capped and heated at 100° for 1 hr. with frequent mixing of the contents. They were then cooled and 1·5 ml. of chloroform–methanol (2:1, v/v) was added and mixed thoroughly with a capillary pipette. The lower layer was removed and the upper layer washed twice with 1 ml. of chloroform–methanol (17:3, v/v), the lower layer being discarded each time. The aqueous upper layer was evaporated to dryness in a stream of N_2 and the residue taken up in precisely 0·15 ml. of water. 4-Amino-4′-chlorobiphenyl reagent (4-amino-4′-chlorobiphenyl hydrochloride, 0·224 g.; cetyltrimethylammonium bromide, 0·10 g. in 100 ml. of 0·1 N-HCl) (0·5 ml.) was added. The tubes were capped, mixed and kept in the refrigerator overnight. They were centrifuged, 0·1 ml. of the supernatant was diluted to 10 ml. with water, and the extinction read at 254 mμ. Duplicate sulphate standards and blanks were carried through the procedure from the point of hydrolysis onwards. Sulphate was calculated from the difference in extinction between the blank and the samples (ΔE).

Sulphate activation

Adenosylsulphatophosphatase was estimated by the method of Wilson & Bandurski (1958). This method depends on the reaction:

$$ATP + SO_4^{2-} \rightleftarrows AMP\text{-}SO_4 + \text{pyrophosphate}$$

moving rapidly to the right in the presence of pyrophosphatase, and is enhanced by the presence of molybdate ions, which form a less stable adenosyl derivative than does sulphate. Inorganic phosphate formed by the pyrophosphatase thus provides a measure of the overall reaction. Brain contains a highly active pyrophosphatase (Gordon, 1950) and addition of yeast pyrophosphatase (cf. Wilson & Bandurski, 1958) was not considered necessary.

Incubation mixtures contained: ATP (0·01 M) and either sodium molybdate (0·01 M) or Na_2SO_4 (0·01 M); $MgCl_2$ (2 mM); tris–HCl buffer (pH 7·4; 0·1 M); sodium ethylenediaminetetra-acetate (0·6 mM); enzyme suspension (0·2 ml.) Total vol., 0·5 ml. Sodium molybdate and sulphate were replaced by chloride for the measurement of adenosine triphosphatase. Incubation was at 37·5° for 30 min. The reaction was stopped by boiling for 2 min. The enzyme suspension consisted of guinea-pig cerebral cortex dispersed in 10–20 vol. of water, sucrose (0·25 M) or NaCl (0·15 M).

Determination of radioactivity

In sulphatides. The precipitated 4-amino-4′-chlorobiphenyl sulphate was taken up in not more than 0·1 ml. of water and plated on polythene disks (1·75 cm.²). After evaporation of the solution to dryness the radioactivity was counted under an end-window counter to an accuracy of 5%. No correction for self-absorption was made, since the quantities of sulphate determined were usually less than 0·5 μmole and over the range 0·3–0·75 μmole of sulphate the recoveries of radioactivity were 99·1% [S.E.M. ±2·65 (9)] without any correction being applied.

In incubation media. A portion (0·1 ml.) of the medium was plated as described above and counted in a similar fashion. Usually the counts were of the order of 2×10^5 counts/min./ml. of medium.

Units. Specific activity = counts/min./μmole of sulphate calculated to a standard count of 10^5 counts/min. in each ml. of the incubation medium.

Reagents

^{35}S was obtained as carrier-free sulphate from The Radiochemical Centre, Amersham, Bucks. 4-Amino-4′-chlorobiphenyl was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. All other reagents were standard laboratory chemicals of AnalaR or P.B.C. quality.

RESULTS

Determination of sulphatide sulphate

At the start of this work no information was available on the effectiveness of the method of Long & Staples (1961) for the quantitative determination of radioactive sulphate, and it was necessary to examine the method from this point of view. As described by Jones & Leytham (1956) and Long & Staples (1961), sulphate is precipitated by the addition of a volume of the reagent equal to that of the sulphate solution. Although this method yielded an apparently straight-line relationship between the concentration of sulphate and ΔE (Fig. 1*A*), when radioactive sulphate was included a similar proportionality between quantity and radioactivity was not obtained (Fig. 1*B*). Presumably the relation between sulphate concentration and ΔE is also a curve under these conditions but this is not easily detectable, unless sulphate is measured by a more sensitive method such as the determination of radioactivity. A straight-line relationship was obtained, however, if the quantity of reagent was doubled (Fig. 1*B*). Under these conditions also, a straight-line relationship existed between the concentration of sulphate and the ΔE, the latter values being higher than those obtained when equal volumes of reagent and sample were used. Recoveries of radioactivity over the range 0·3–0·75 μmole of sulphate were 99·1 ± 2·65% (9 determinations). To achieve this, samples were kept for 16–18 hr. at 2°. Shorter periods, 2–3 hr. (Jones & Leytham, 1956), yielded recoveries of only 76·0 (S.D. of the mean ± 3·2)% (6 determinations). Owing to the extremely low solubility of the sulphate (Belcher, Nutter & Stephen, 1953) the lines relating sulphate concentration to ΔE pass through the origin. Quantities above 0·8 μmole of sulphate were not tested, being greater than any encountered with slices of cerebral tissue.

Under the conditions examined orthophosphate up to 4·0 μmoles or β-glycerophosphate up to 10 μmoles did not interfere with the determination of sulphate. Addition of radioactive phosphate showed that contamination by orthophosphate, present in a concentration of 6·6 mM, did not exceed 3%. Heating β-glycerophosphate in the presence of sulphate, under the conditions used for the hydrolysis of sulphatides, did not affect the recovery of sulphate. It was concluded that the major phosphorylated products likely to be released during acid hydrolysis of the lipid extract would not interfere with the determination of sulphate.

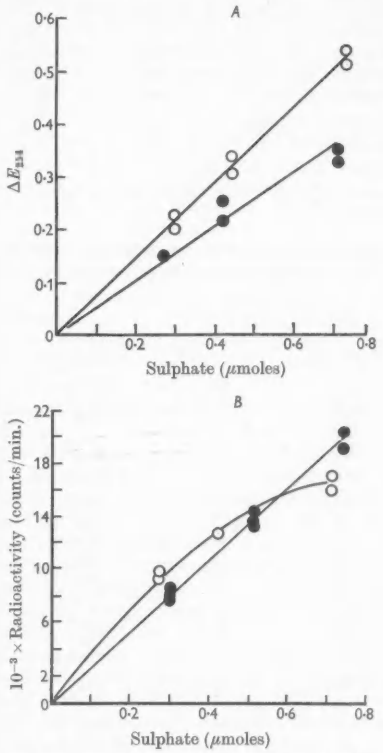

Fig. 1. The precipitation of radioactive sulphate from 0·15 ml. of aqueous solution by different quantities of 4-amino-4-chlorobiphenyl reagent. For experimental details and method of estimating ΔE see the Materials and Methods section. *A*, The relationship between ΔE and quantities of sulphate: ○, with 0·30 ml. of reagent; ●, with 0·15 ml. of reagent. *B*, The relation between radioactivity of the precipitated sulphate and the quantity originally present in solution: ●, 0·30 ml. of reagent added; ○, 0·15 ml. of reagent added.

With the quantities of radioactive sulphate employed (40 μc/vessel) it was estimated that 0·1 ml. of solution, or 15 000–20 000 counts/min., would be carried over with the slices into the chloroform–methanol. Radioactive carrier-free sulphate added to the tissue extracts was quantitatively removed by the washing procedure of Folch *et al.* (1957). More than 95% of the radioactivity was removed in the first two washings and the remainder by the third and fourth washings. Since this method has been shown to leave virtually all the tissue lipids in the chloroform-rich lower phase (Folch *et al.* 1957), it was concluded that the sulphatides could be adequately separated from radioactive sulphate carried over from the incubation medium. Radioactive sulphate added to the lipid extracts immediately before hydrolysis and carried through the remaining procedure was quantitatively recovered. Thus in four experiments with lipids extracted from 400 mg. of tissue the recoveries were 96·5 ± 4·5% (S.E.M.).

Metabolic experiments

Initial experiments carried out in salines containing 1·28 mM-sulphate showed that the radioactivity incorporated was too small to be measured by the methods used. A measurable amount was incorporated if the medium was free from added sulphate. Under these conditions the degree of incorporation into the sulphur-containing lipids was not altered by the buffer used. Thus, in saline media containing glucose, replacement of the buffer tris–HCl by sodium phosphate (pH 7·4; 0·05M) or glycylglycine–HCl (pH 7·4, 0·05M) produced little variation in the radioactivity incorporated into the sulpholipids. Radioactive sulphate was also incorporated into the lipid-free residue, which presumably contains chondroitin sulphate (Brante, 1957). Attempts to determine the ester sulphate in this fraction were unsuccessful and further examination was not attempted.

Table 1. *Effects of anaerobiosis and lack of glucose on the incorporation of* ^{35}S *into sulphatides of guinea-pig cerebral slices*

Slices were cut, weighed and incubated under conditions described in Materials and Methods. Glucose was included where stated. All vessels contained 40 μc of ^{35}S from the start of the experiment. Incubation was for 2 hr. at 37·5°. Specific activity = counts/min./μmole of sulphate calculated to a standard count of 10^5 counts/min./ml. of medium.

Conditions	Gas phase	Specific activity	
		Expt. 1	Expt. 2
Glucose (10 mM)	O_2	1160	1930
Glucose (10 mM)	N_2	9·6	231
No glucose	O_2	Not estimated	314

Table 1 shows that both substrate and oxygen were necessary for maximal incorporation of ^{35}S into sulphatides. Incorporation was markedly reduced by the presence of 2:4-dinitrophenol (Table 2). This occurred both at concentrations (6×10^{-4} M) sufficient to decrease the concentrations of adenosine triphosphate and phosphocreatine in the tissues (Kratzing & Narayanaswami, 1953) and at lower concentrations where levels of phosphocreatine only are decreased.

The effect of various substrates in supporting sulphate incorporation into the sulphatides is shown in Table 3. In these experiments the various substrates were compared for their effectiveness with slices incubated both in the absence of additional substrate and in the presence of glucose. Of the metabolites examined only pyruvate was able both to maintain a respiration and an incorporation of sulphate equivalent to glucose. Mannose and fructose, though maintaining respiration and supporting sulphate incorporation, did so to a less degree than did glucose. Succinate even at concentrations maintaining a high respiration was almost without effect upon sulphate incorporation, as were galactose, glutamate, citrate and fumarate.

Table 2. *Effect of 2:4-dinitrophenol on the incorporation of ^{35}S into sulphatides of guinea-pig cerebral cortex*

Slices were incubated as described in Materials and Methods. Glucose was included in the saline. Dinitrophenol was added from the side arm after equilibration of the vessels for 10 min. at 37·5°.

Concn. of 2:4-dinitrophenol	Oxygen uptake (μmoles/g. wet wt.) 0–60 min.	60–120 min.	Specific radioactivity
0	58	57	966
0·6 mM	74	48	595
0	62	62	5250
6 μM	122	126	1580
0	60	60	2320
6 μM	80	80	1180
60 μM	108	108	730

Table 3. *Incorporation of ^{35}S into sulphatides of guinea-pig-brain slices when metabolizing in the presence of different substrates*

Experimental conditions and determination of sulphatides were as described in Materials and Methods. Specific activity = counts/min./μmole of sulphate calculated to a standard count of 10^5 counts/min./ml. of medium.

Expt. no.	Substrate	Concn. (mM)	Oxygen uptake (μmoles/g. wet wt.) 0–60 min.	60–120 min.	Specific activity of sulphate
1	Glucose	10	72	72	916
	Mannose	10	65	65	485
	Fructose	10	61	61	610
2	No substrate	—	33	15	364
	Mannose	10	65	65	1870
	Fructose	10	64	64	2590
3	Glucose	10	66	66	560
	Galactose	10	49	21	172
	Succinate	10	75	40	295
4	No substrate	—	42	17	264
	Galactose	10	57	25	210
	Succinate	10	61	39	374
5	Glucose	10	63	63	2400
	Pyruvate	20	55	55	2120
	Glutamate	10	72	58	550
6	No substrate	—	33	16	184
	Glutamate	10	60	37	121
7	Glucose	10	61	61	2600
	Citrate	20	36	14	475
8	No substrate	—	40	20	575
	Pyruvate	20	66	66	1255
	Citrate	20	42	21	666
9	Glucose	10	61	61	1300
	Fumarate	20	45	29	300
10	No substrate	—	50	32	845
	Fumarate	20	50	35	740
11	Glucose	10	67	67	1030
	Succinate	10	56	45	373
	Succinate	50	108	48	243

Table 4. *Sulphate activation by dispersions of guinea-pig cerebral cortex*

For experimental details see the Materials and Methods section. Values are μmoles of inorganic phosphate released and are corrected for adenosine-triphosphatase activity.

	Tissue equivalent (mg.)	Phosphate liberated (μmoles)		Phosphate liberated (μmoles/g. wet wt./hr.)	
		With MoO_4^{2-}	With SO_4^{2-}	With MoO_4^{2-}	With SO_4^{2-}
Water dispersion	2·5	0·39	—	78·0	—
Water dispersion	12·5	—	0·73	—	29·2
Water dispersion	12·5	1·83	0·82	73·2	32·8
Dispersion in 0·25 M-sucrose	12·5	3·53	—	157·0	—
Dispersion in 0·15 M-NaCl	8·0	0·42	0·15	105·0	38·0

Rate of metabolism of sulphate by brain tissue. The radioactivity of sulphatide sulphate obtained from 200 mg. of slices incubated for 2 hr. with glucose was generally between 400 and 600 counts/min. These values were equivalent to 0·08–0·12 μc of ^{35}S (carrier-free) counted under the same conditions and in the same apparatus. Since each slice was incubated with 40 μc of ^{35}S and the sulphatides from two slices were pooled for analysis, these quantities of 0·08–0·12 μc represented no more than 0·1–0·15% of the sulphate added. Although this level of incorporation was similar to that described for gastric mucins (Pasternak, Kent & Davies, 1958), it seemed possible that the low incorporation was due to a low permeability of the tissue slices to sulphate rather than to a low rate of metabolism.

In an attempt to decide the potential rate at which sulphate could be metabolized by brain dispersions, the rate of sulphate activation by guinea-pig cerebral cortex was measured. The results are presented in Table 4 and show the speed of the adenosine triphosphate-sulphurylase reaction. Since the product of the reaction is highly unstable when molybdate replaces sulphate, the rate of the reaction in the presence of this anion is measurably greater (Wilson & Bandurski, 1958). Even so, the speed of reaction in the presence of sulphate (30–38·0 μmoles of phosphate released/g. wet wt. of tissue/hr.) was considerable. Since the inorganic phosphate determined is liberated by pyrophosphatase from pyrophosphate produced during sulphate activation, the values represent speeds of reaction of 39–78·0 μmoles/g. wet wt./hr. in the presence of molybdate and 15–19 μmoles/g. wet wt./hr. in the presence of sulphate.

Incubation of sulphatide A from ox brain in this system in the presence of radioactive sulphate did not result in any measurable incorporation of radioactivity into the sulpholipid fraction finally obtained. This was so whether the incubation was with an aqueous dispersion or with a dispersion made in iso-osmotic sucrose. This result could not be attributed to breakdown of sulphatide by sulphatases in the dispersion since incubation of 1·3 μmoles of sulphatide A with 50 mg. of tissue dispersion either unbuffered in water or buffered at pH 5·9 with sodium acetate–acetic acid (0·125 M) did not release any measurable quantities of sulphate into the solution. Sulphate added to this system was quantitatively recovered. It was concluded that under these conditions added sulphatide is not metabolized.

DISCUSSION

It has been shown that slices of cerebral cortex from the adult guinea pig, when respiring in a medium containing glucose, incorporate inorganic sulphate added to the medium into sulpholipids. The sulpholipids involved have not been isolated, but it seems probable that the results reported here concern a single major substance. Thus although the sulphatides isolated by Blix (1933) and Thannhauser *et al.* (1955) represented only a small sample of the total sulpholipid sulphur, this has been shown to be due to the methods employed. Using a simple technique Lees *et al.* (1959) have convincingly demonstrated that a sulphatide, 'sulphatide A', could be readily obtained in a high yield and that an associated sulphatide (sulphatide B) accounted for the remaining sulpholipid sulphur. Chemically, the two were not distinguishable. More recently Green & Robinson (1960) showed that only one radioactive sulphatide could be isolated from extracts of rat brain after injection of radioactive sulphate *in vivo*. In human brain, however, two sulphatides can be distinguished, differing in the fatty acid components of the molecule (Jakewitz, 1960*a*). Similar fractions from the brain of other mammals have not been reported.

Incorporation of sulphate into the sulphatides was inhibited by 2:4-dinitrophenol, an uncoupler of oxidative phosphorylation (Table 2). Further, substrates that were unable to support sulphate incorporation are similar to those unable to support adequate levels of energy-rich phosphates such as phosphocreatine in cerebral slices (Heald, 1960), and suggest that a common factor may be involved.

Of several, the simplest would appear to be adenosine triphosphate. Since sulphate activation, generally, involves adenosylsulphatophosphate formation (Gregory & Robbins, 1960) the inability of certain substrates to maintain adequate levels of energy-rich phosphates in brain slices would appear to present a plausible explanation of their failure to support sulphate incorporation. However, it seems likely that other factors are also involved. Thus the ability of pyruvate to support the incorporation of sulphate (Table 3) is not paralleled by its ability to support the incorporation of phosphate into phospholipids, whereas fructose, supporting sulphate incorporation to an extent greater than mannose (Table 3), was much less effective in supporting phosphate incorporation into phospholipids (Findlay, Rossiter & Strickland, 1953; Findlay, Magee & Rossiter, 1954). Since phosphate incorporation into phospholipids also involves adenosine triphosphate these differing effects of substrates may reflect the ability or otherwise of the substrate to synthesize sulpholipid precursors.

Although dispersions of cerebral tissue contain adenosine triphosphate sulphurylase acting at a rate of up to 19 μmoles of sulphate/g. wet wt./hr., no sulphate was incorporated into added sulpholipid under similar conditions. Although the formation of adenosine 3′-phosphate 5′-sulphatophosphate was not measured there is little reason to doubt that tissues containing adenosine triphosphate sulphurylase are capable of forming it (Gregory & Robbins, 1960). Failure to incorporate sulphate into sulphatide A may therefore imply either a loss of or absence of a sulphokinase or that the sulphatide was not a suitable acceptor. Sulphatide A itself was not degraded by dispersions of whole brain, to yield any measurable quantities of sulphate. This latter result is in agreement with those of Green & Robinson (1960), who were similarly unable to demonstrate any release of sulphate from added sulphatide 'A' on incubation with brain dispersions for 3 hr. In the intact tissue different conditions may obtain, for the concentrations of sulphatide sulphur in slices incubated for 2 hr. with glucose were ($\pm$S.E.M.) $0{\cdot}84 \pm 0{\cdot}05$ (28 determinations) μmole of sulphate/g. wet wt. of tissue, and without added substrate $0{\cdot}61 \pm 0{\cdot}11$ (6) μmole of sulphate/g. wet wt.

The results appear to show that the sulphatides of brain tissue, once formed, are capable of little further metabolic transformations. This applies *in vivo* and extends to the galactose fragment of the sulphatide. Thus, after the injection of the [^{14}C]-galactose or $^{35}SO_4^{2-}$ into both young and adult rats, the radioactivity of the sulpholipids isolated from the brain reached a peak in 3 days and thereafter remained constant for at least 16 days (Radin *et al.* 1957). Green & Robinson (1960) and Ringertz (1956) found that the decrease in radioactivity of the brain sulpholipids, labelled by intraperitoneal injection, persisted for up to several weeks. In the absence of any demonstrable sulphatidase activity in brain dispersions it is considered that incorporation of sulphate into sulphatides in brain represents the slow synthesis of new sulpholipid and is not simply an exchange of existing sulphate.

The extremely slow metabolism of the sulphatides parallels that described by Davison & Dobbing (1960) for certain cerebral phospholipids and also for cholesterol (Davison, Dobbing, Morgan & Payling-Wright, 1959), both of which substances are considered to be components of the myelin sheath. The precise localization of the sulpholipids in brain is not clear, a distribution between both the 'mitochondrial' and 'nuclear' fractions having been found by Green & Robinson (1960). The early work of Koch & Koch (1913) in which the maximum increase of sulpholipids coincided with the period of maximum myelination has also been interpreted to indicate a localization in the myelin sheath and much of the metachromatic staining properties of the sheath have been attributed to the presence of sulphatides (Diezel, 1957). Also, in the Scholtz leucodistrophy degeneration of the myelin is accompanied by a marked increase in the sulphatide (Jakewitz, 1960*a*, *b*). In so far as the properties of such a structure require a degree of metabolic stability of its components, the apparent metabolic inertness of the sulphatides would not be inconsistent with a location in the myelin sheath.

SUMMARY

1. The incorporation of radioactive sulphate into the sulphatides of guinea-pig cerebral slices is dependent upon oxidative phosphorylation. Glucose or pyruvate was essential for maximum incorporation; mannose and fructose were less effective than glucose, while succinate, citrate, fumarate and glutamate did not support incorporation.

2. Dispersions of whole brain activated sulphate via the adenosylsulphatophosphate system at rates up to 20 μmoles/g. wet wt. of tissue/hr. Sulphatides added to this system containing $^{35}SO_4^{2-}$ did not exchange sulphate, nor was any sulphate liberated from added sulphatide by incubation for up to 24 hr. In the intact slice the incorporation of radioactive sulphate in 2 hr. amounted to only 0·1–0·15 % of the sulphate in the medium.

3. It is concluded that the sulphatides of brain are metabolically inert and may be localized in the myelin sheath. The incorporation of sulphate into the sulphatides of the brain slices is considered to be the result of a total synthesis of new sulphatide.

It is a pleasure to thank Dr C. Long for giving details of the method of sulphatide analysis before publication, and Dr G. H. Sloane-Stanley for helpful discussions on the washing procedure used for removal of radioactive sulphate and for a gift of sulphatide A.

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Stimulation of Ascorbic Acid Synthesis and Excretion by Carcinogenic and other Foreign Compounds

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Increases in the hepatic ascorbic acid concentrations of mice occur after a single injection of a variety of carcinogenic hydrocarbons (Kennaway, Kennaway & Warren, 1944; Daff, Hoch-Ligeti, Kennaway & Tipler, 1948) although experiments with smaller doses of some carcinogenic compounds had not shown such increases (Boyland & Mawson, 1938).

Longenecker, Fricke & King (1940) demonstrated that narcotics such as chloretone raise the excretion of ascorbic acid in rats and considered that this was associated with an increase in detoxication processes. Chloretone or barbital treatment stimulates the biosynthesis of glucuronic acid and ascorbic acid (Burns & Evans, 1956; Burns, Evans & Trousof, 1957; Evans, Conney, Trousof & Burns, 1960). This increase is abolished by hypophysectomy but not by adrenalectomy (Conney, Gastel & Burns, 1959), nor is it an effect of the drug on the mechanism for the renal tubular reabsorption of ascorbic acid since it can also be produced in nephrectomized rats (Burns *et al.* 1960). Increase in ascorbic acid excretion also occurs after the injection of aminopyrine, antipyrine, diphenhydramine, chlorcyclizine, orphenadrine, meprobamate and phenylbutazone (Burns *et al.* 1960). These authors suggest that the raised biosynthesis of ascorbic acid that takes place after an injection of a wide variety of chemical compounds represents an adaptive response to foreign compounds although borneol and aniline, which are conjugated with glucuronic acid, do not affect ascorbic acid excretion. An increase in ascorbic acid excretion after treatment with carcinogenic hydrocarbons has

been noted by Allen & Boyland (1957) and by Conney *et al.* (1959).

The precise mechanism by which foreign compounds affect glucuronic and ascorbic acid biosynthesis is not known, but the response to some foreign substances is not restricted entirely to an increase in ascorbic acid production; increases in the activity of hepatic microsomal enzymes that metabolize foreign substances also occur after the administration of some hypnotics and carcinogenic hydrocarbons (Conney, Miller & Miller, 1956; Conney *et al.* 1959; Inscoe & Axelrod, 1960).

The present work describes the effect on ascorbic acid metabolism of a variety of foreign compounds.

MATERIALS AND METHODS

D-Galacturonic acid was purchased from L. Light and Co. Ltd., Colnbrook, Bucks., and the sodium salt of 2:6-dichlorophenol-indophenol from Hopkin and Williams Ltd. The extracts of quebracho and mimosa were gifts from Dr K. S. Kirby. Other chemicals used were of analytical grade; reagents were prepared in water distilled in glass. Dextran C and Imferon were from Benger's Ltd., Holmes Chapel, Cheshire.

Slonaker rats were obtained from Dr A. L. Walpole, Imperial Chemical Industries (Pharmaceutical Division) Ltd., Alderley Park, Cheshire.

Preparation of L-*galactono-γ-lactone.* D-Galacturonic acid (4 g.) was dissolved in methanol (100 ml.), Raney Ni catalyst (1·0 g.) was added, and the mixture heated at 100° for 24 hr. under a pressure of 150 atm. of H_2 in a Baskerville rocking autoclave. The cooled mixture was treated with activated charcoal and filtered. The filtrate was evaporated to dryness under reduced pressure, and the syrup transferred, with the aid of a little hot methanol, to a dish which was kept *in vacuo* over conc. H_2SO_4 for 48 hr. The white waxy solid (3·2 g.) was crystallized from absolute ethanol–ether (5:1, v/v) to give 2·1 g. of the lactone (55% of theory), m.p. 128°.

Excretion of ascorbic acid. Pairs of 200 g. male rats were housed in metabolism cages designed for the separate collection of urine and faeces and were maintained on a constant 20% protein diet throughout. All-glass urine separators were used and urine collections were made in flasks containing 10 ml. of aq. metaphosphoric acid (12%, w/v).

Estimation of ascorbic acid. This was estimated by direct titration with a solution of the sodium salt of 2:6-dichlorophenol-indophenol (Harris & Olliver, 1942). In some experiments 'total' ascorbic acid, which includes dehydroascorbic acid and 2:3-dioxogulonic acid, was determined by the method of Roe & Kuether (1943) by using 2:4-dinitrophenylhydrazine.

Identification of 2:6-*dichlorophenol-indophenol-reducing substance.* The indophenol-reducing substances present in rat urine and produced by rat-liver microsomal enzymes were examined by paper chromatography. Samples of urine and enzyme incubation mixtures were chromatographed on Whatman no. 1 paper that had previously been treated with metaphosphoric acid (1%, w/v) by dipping (Mitchell & Patterson, 1953). The solvent system used was the upper layer of freshly prepared butan-1-ol–acetic acid–water (4:1:5, by vol.; Partridge, 1948). The descending chromatograms were run in the presence of NaCN (Mapson & Partridge, 1949) and, when dry, were sprayed with either a solution of 2:6-dichlorophenol-indophenol or with ammoniacal $AgNO_3$ (Mapson & Partridge, 1949). The only indophenol-reducing substance found had the same R_F as authentic L-ascorbic acid (0·39). Dehydroascorbic acid (R_F 0·48) was also identified in rat urines.

Preparation of microsomal fraction of rat liver. Rat liver from freshly killed animals was homogenized in 4 vol. of ice-cold 0·25M-sucrose (Schneider, 1948) and the homogenate centrifuged at 900*g* in a refrigerated centrifuge to remove cell debris. The supernatant was recentrifuged at 20 000*g* for 10 min. to remove mitochondria and the microsomes were then aggregated by centrifuging at 90 000*g* for 40 min. in a Spinco model L preparative ultracentrifuge. The microsomes were washed by resuspending them in ice-cold 0·25M-tris buffer (pH 7·5) followed by centrifuging at 90 000*g* for 40 min. The temperature of the preparations was maintained between 0° and 5° and prepared microsomes were stored for up to 48 hr. at −20°.

Microsomal ascorbic acid synthesis. For ascorbic acid synthesis a typical incubation mixture contained the microsomes from about 0·7 g. of liver resuspended in 0·1M-sodium phosphate buffer (pH 7·4) containing 0·13M-sucrose (2·0 ml.) and a freshly prepared solution of L-galactono-γ-lactone (2·5 mg.), 1:1:1-trichloro-2-methylpropan-2-ol (2·0 mg.; Isherwood, Mapson & Chen, 1960) and $Na_4P_2O_7$ (18·0 mg.; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1960) in 0·1M-phosphate buffer (pH 7·4) containing 0·25M-sucrose (6·0 ml.). The mixture was incubated for 1 hr. at 37° (Suzuki, Mano & Shimazono, 1960) with shaking in the presence of air; boiled microsomes were used as a blank. After incubation the enzyme was inactivated by the addition of metaphosphoric acid (12%, w/v; 2·0 ml.) and portions were titrated with 2:6-dichlorophenol-indophenol.

RESULTS AND DISCUSSION

Urinary excretion of ascorbic acid

The normal daily excretion of ascorbic acid by pairs of rats was determined and thereafter for 16 days after a single injection of a compound. Intraperitoneal injection of 1:2-5:6-dibenzanthracene (dibenz[*a*, *h*]anthracene) (50 mg./kg.) increased both the 'total' and 'indophenol-reducing' ascorbic acid excretion of two male rats (Fig. 1). The values obtained by titration with 2:6-dichlorophenol-indophenol were lower but parallel with those for 'total' ascorbic acid; in further experiments titration with 2:6-dichlorophenol-indophenol alone was used to measure ascorbic acid excretion.

The non-carcinogenic hydrocarbons, phenanthrene and anthracene, gave no significant increase in ascorbic acid excretion (Table 1). The 14-fold increase in ascorbic acid excretion produced by 50 mg./kg. of 1:2-5:6-dibenzanthracene was decreased when 20 mg./kg. of 2-diethylaminoethyl 2:2-diphenylvalerate hydrochloride (SKF 525A)

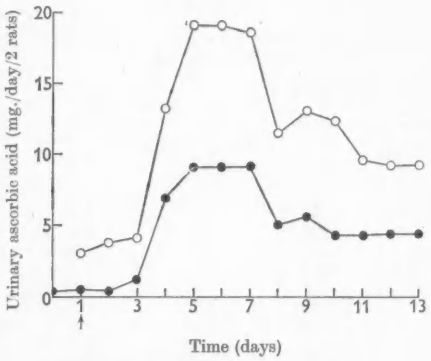

Fig. 1. Urinary ascorbic acid excretion measured by titration with 2:6-dichlorophenol-indophenol (●) and by formation of 2:4-dinitrophenylhydrazone (○) after an intraperitoneal injection (↑) of 1:2-5:6-dibenzanthracene (50 mg./kg.) to two male Wistar rats.

was injected at the same time. This compound (Brodie *et al.* 1955) inhibits the metabolism of drugs by a variety of pathways and it affects particularly microsomal systems, the main site for hepatic ascorbic acid synthesis (Mapson, 1960). 3-Methylcholanthrene gave a smaller increase in ascorbic acid excretion than has been reported by Conney *et al.* (1959). Inscoe & Axelrod (1960) found that treatment of rats with 3:4-benzopyrene (benzo[*a*]-pyrene) increased microsomal glucuronide synthesis *in vitro* by 40% in males and over 250% in females.

The liver carcinogens 4-dimethylaminoazobenzene and 3′-methyl-4-dimethylaminoazobenzene increased excretion of ascorbic acid as did injection of the non-carcinogenic isomeric 2-methyl-4-dimethylaminoazobenzene and 4′-methyl-4-dimethylaminoazobenzene (Table 1). Dimethylnitrosamine, which Magee & Barnes (1956) have shown to produce hepatic tumours in rats, was without effect on ascorbic acid excretion, whereas bromobenzene and to a lesser extent carbon tetrachloride, both of which produce liver necrosis (Popper, de la Huerga & Koch-Weser, 1954; Lamson & Wing, 1926), increased ascorbic acid excretion. Thioacetamide and ethionine, which produce toxic liver injury (Gupta, 1956; Popper *et al.* 1954) and are also carcinogenic (Fitzhugh & Nelson, 1948; Popper, de la Huerga & Yesinick, 1953), increased ascorbic acid excretion.

The injection of a solution of tannic acid, which Korpassy (1959) has shown to have both a necrotic and a hepatocarcinogenic action, gave no increase in ascorbic acid excretion, nor did two tannic acid-containing extracts, quebracho and mimosa, which Kirby (1960) has found to cause liver cancer.

The effects on urinary ascorbic acid concentrations of injections of lead acetate, which causes kidney tumours (Zollinger, 1953), were not conclusive (Table 1) but some delayed increase in excretion was apparent. Treatment with 4-amino-4′-fluorobiphenyl, which also causes kidney tumours (Matthews & Walpole, 1958), gave an immediate increase in ascorbic acid excretion. A more marked increase was produced by a similar dose of 4-aminobiphenyl, which Melick, Escue, Naryka, Mezera & Wheeler (1955) have found to cause cancer of the bladder in man. Although dextran C, which does not induce cancer on injection into rats, gave an increase in urinary ascorbic acid, the iron–dextran complex Imferon, which Richmond (1959) found to cause cancer at the site of injection, had no effect on ascorbic acid excretion. 2-Acetamidofluorene has, amongst other carcinogenic properties, been reported to produce tumours of the urinary tract in the Slonaker strain of rats but not in Wistar rats (Wilson, De Eds & Cox, 1941; Bielschowsky, 1944). The urinary ascorbic acid excretion level was increased in both of these two strains, but the increase was greater in the Wistar rats (Table 1). Injection of sodium arsenite was without effect on urinary ascorbic acid.

The enhanced excretion of ascorbic acid produced by injection of the hypnotic 2-*iso*propyl-4-pentenoyl)urea (Sedormid) and the non-hypnotic allyl-*iso*propylacetamide (Table 1) is similar to that found to occur with other narcotic compounds (Longenecker *et al.* 1940; Burns *et al.* 1960). In addition to producing liver necrosis Sedormid and allyl*iso*propylacetamide cause porphyrinuria (Goldberg & Rimington, 1954). The injection of Tween 80 gave a delayed increase in urinary ascorbic acid excretion. Nitrogen mustard produced variable responses but neither croton oil nor chlorambucil increased urinary ascorbic acid excretion.

Ascorbic acid biosynthesis by rat-liver microsomes

The increased urinary excretion of ascorbic acid which occurs after treatment of rats with a wide variety of compounds has been attributed to an increase in microsomal ascorbic acid synthesis by the glucuronic acid pathway (Burns *et al.* 1960). After establishing normal values for the microsomal synthesis and the urinary excretion of ascorbic acid in a group of male rats, 12 were injected with 1:2-5:6-dibenzanthracene (50 mg./kg.) which has been shown (Table 1) to increase ascorbic acid excretion. The daily urinary excretion of one pair of rats was followed; the others were killed in pairs at intervals after injection. Microsomes were prepared from the homogenized livers and the level of microsomal ascorbic acid synthesis was determined.

Table 1. *Effect of foreign compounds on urinary ascorbic acid excretion in the rat*

Except where stated, male Wistar rats (200 g.) were used and compounds were administered intraperitoneally. Figures in parentheses refer to the day after the injection on which the highest value was recorded.

Compound	Dose (mg./kg.)	Mean ascorbic acid excretion (mg./day/2 rats)		Highest values (mg./day/2 rats)	
		Before injection (8 days)	After injection (16 days)	Before injection	After injection
1:2-5:6-Dibenzanthracene	1·0	1·04	0·67	1·90	1·44 (9)
	2·0	0·69	0·69	1·40	2·08 (9)
	5·0	0·47	0·38	1·14	0·74 (11)
	5·0	0·26	0·51	0·81	1·43 (9)
	5·0*	0·22	0·24	0·51	0·72 (4)
	10·0	0·30	0·49	0·57	1·36 (5)
	10·0†	0·97	2·12	1·59	6·24 (8)
	20	0·43	0·68	1·25	1·97 (7)
	50	0·69	4·96	1·18	10·00 (2)
	50‡	0·45	2·68	0·69	8·00 (10)
	50§	0·76	2·43	1·47	4·50 (2)
1:2-5:6-Dibenzanthracene	50	—	—	—	—
SKF 525A	20	0·71	2·31	1·66	4·00 (11)
Anthracene	100	0·60	0·52	1·00	1·12 (9)
Phenanthrene	100	0·26	0·35	0·35	1·18 (4)
3-Methylcholanthrene	20	0·53	1·29	0·96	2·19 (6)
4-Dimethylaminoazobenzene	100	1·24	1·49	1·56	3·70 (8)
3′-Methyl-4-dimethylaminoazobenzene	100	0·99	1·48	1·70	3·84 (16)
2-Methyl-4-dimethylaminoazobenzene	100	0·71	1·25	1·26	2·08 (10)
4′-Methyl-4-dimethylaminoazobenzene	100	0·44	1·12	0·71	2·86 (9)
Dimethylnitrosamine	15	0·25	0·29	0·50	0·92 (5)
Bromobenzene	500	0·85	0·90	1·50	6·25 (1)
Carbon tetrachloride	1·0‖	0·44	0·58	0·55	1·70 (1)
Thioacetamide	100	0·92	1·01	1·85	5·26 (1)
Ethionine	250	0·23	0·41	0·28	1·11 (2)
Tannic acid	100	0·29	0·44	0·61	1·00 (12)
Quebracho extract	100	0·57	0·79	0·98	1·52 (15)
Mimosa extract	100	0·68	0·60	0·91	1·12 (7)
Lead acetate	50	0·88	0·80	1·43	2·56 (8)
	50	1·36	2·38	1·82	5·27 (7)
4-Amino-4′-fluorobiphenyl	100	0·79	0·64	1·25	2·18 (1)
4-Aminobiphenyl	100	0·89	1·13	1·47	4·17 (1)
Dextran C	5·0‖	0·30	1·15	0·42	2·44 (12)
Imferon	2·5‖	0·21	0·77	0·42	1·67 (10)
	2·5‖	0·32	0·40	0·56	1·04 (15)
	5·0‖	1·22	0·93	1·96	2·34 (3)
	10·0‖	1·26	0·37	1·89	0·72 (7)
2-Acetamidofluorene	100	0·59	1·17	0·93	2·70 (4)
	100†	0·43	0·53	0·67	1·47 (1)
Sodium arsenite	5·0	1·23	0·41	1·92	0·86 (2)
Sedormid	100	0·19	0·67	0·24	2·38 (1)
Allyl*iso*propylacetamide	100	1·00	0·99	1·29	5·55 (1)
Croton oil	25	0·38	0·43	1·00	0·74 (6)
Methylbis(2-chloroethyl)amine	0·5	0·25	0·27	0·29	0·57 (16)
	1·0	0·35	0·38	1·35	1·43 (1)
	1·0	0·39	1·09	0·52	2·34 (16)
Chlorambucil	10·0	0·49	0·60	1·21	1·05 (7)
Tween 80	2·5†	0·67	0·76	1·15	2·04 (12)
	5·0†	1·18	1·23	1·67	3·85 (9)

* Administered subcutaneously. † Male Slonaker rats (200 g.). ‡ Female Wistar rats (200 g.).
§ Male Wistar rats (50 g.). ‖ ml./kg.

Whilst an eightfold increase in urinary ascorbic acid had occurred by the fifth day after injection, only a small increase in microsomal ascorbic acid synthesis was detected.

After 1:2-5:6-dibenzanthracene treatment urinary ascorbic acid excretion by a pair of male rats rose from an average of 1·25 mg./day before treatment to a mean figure of 4·37 mg./day for the 15 days after the injection (Fig. 2). Burns, Mosbach & Shulenberg (1954) have found, using L-[1-^{14}C]ascorbic acid, that the turnover rate for ascorbic acid was 10·7 mg./100 g. body wt. in

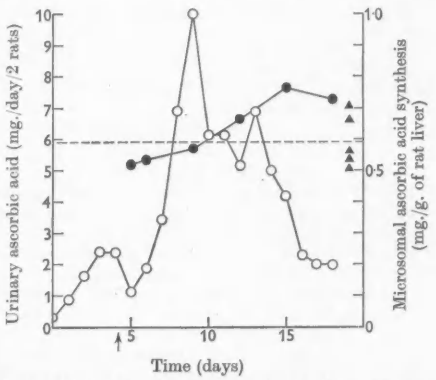

Fig. 2. Effect of an intraperitoneal injection of 1:2-5:6-dibenzanthracene (50 mg./kg.) (↑) on the microsomal synthesis *in vitro* from L-galactono-γ-lactone (●) and the urinary excretion (○) of ascorbic acid by male rats. The level of ascorbic acid synthesis by liver microsomes from pairs of normal rats is shown (▲) (mean for 10 rats – – –). Ascorbic acid was determined by titration with 2:6-dichlorophenol-indophenol.

normal rats and that this was increased in Chloretone-treated rats to 21·5 mg./100 g. body wt. In the present studies on microsomes *in vitro* 0·6 mg. of ascorbic acid/g. of liver/hr. was synthesized from L-galactono-γ-lactone, which is equivalent to a production of 86 mg./100 g. body wt. The high rate of ascorbic acid synthesis *in vitro* suggests that the speed of conversion of L-galactono-γ-lactone into ascorbic acid was not a limiting factor in ascorbic acid biosynthesis *in vivo*. This could explain why only a small increase in microsomal ascorbic acid formation was found after dibenzanthracene treatment.

The drug-induced increase in ascorbic acid formation is apparently brought about by some change other than an increase in the activity of the microsomal enzyme which converts L-galactono-γ-lactone into ascorbic acid. This agrees with the finding of Burns *et al.* (1960) that pretreatment with a compound which increases ascorbic acid excretion results in an increase in the metabolism of glucose through the glucuronic acid pathway, the ascorbic acid being formed from the further metabolism of glucuronic acid. Kanda & Sakamoto (1960) reported that although an increased excretion of ascorbic acid occurs in alloxan-diabetic rats, ascorbic acid breakdown by a hepatic system *in vitro* is decreased. The decrease, which is abolished by insulin treatment, does not occur in barbital-treated rats. Isherwood *et al.* (1960) have shown that the addition of chloral hydrate to rat-liver-microsome preparations increases the rate of ascorbic acid synthesis from L-gulono-γ-lactone by inhibiting the enzymic hydrolysis of this lactone. The rate of ascorbic acid synthesis *in vivo* may be controlled in part by regulation of the amount of substrate available for conversion into ascorbic acid, and some of the varied compounds that accelerate ascorbic acid synthesis in the rat may act by inhibiting enzymic hydrolysis of L-gulono-γ-lactone.

SUMMARY

1. Some foreign compounds increase synthesis and excretion of ascorbic acid in rats.

2. A new method for the preparation of L-galactono-γ-lactone by high-pressure catalytic reduction of D-galacturonic acid is described. The rate of conversion of L-galactono-γ-lactone into ascorbic acid was the same in microsomes from normal rats and rats treated with 1:2-5:6-dibenzanthracene, which caused increased ascorbic acid excretion in the whole animal.

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Studies on Incorporation of Amino Acids into Protein in Isolated Rat-Liver Ribosomes

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Cells contain microsomal particles, or ribosomes (Roberts, 1958), 10–25 mμ in diameter, which contain ribonucleoprotein on which, it is generally believed, amino acids, activated by reactions occurring in the cell sap (Hoagland, Keller & Zamecnik, 1956; Hoagland, Zamecnik & Stephenson, 1957), are assembled into polypeptide chains during the biosynthesis of proteins (see Hoagland 1960, for review). This view is greatly strengthened by the results obtained by Littlefield, Keller, Gross & Zamecnik (1955) in experiments with whole animals, which are consistent with the hypothesis that proteins are formed in the ribosomes and are transferred, via the lipoprotein of the endoplasmic reticulum, to the soluble fraction of the cell.

In the experiments of Littlefield *et al.* (1955) ribosomes were prepared from microsomes by treatment of the latter with sodium deoxycholate (Palade & Siekevitz, 1956), which solubilizes the lipoprotein part of the microsomes (Strittmatter & Ball, 1952). The ribosomes were reported (Littlefield & Keller, 1957) to be incapable of incorporating radioactive amino acids into protein *in vitro* in a system of the type described by Zamecnik & Keller (1954). The implication was that treatment with deoxycholate may have damaged the ribosomes.

Ribosomes from many tissues have been intensively studied recently and their ability to aggregate and disperse in a manner dependent on the pH and composition of the suspending medium and in particular on the concentration of magnesium ions in the medium has excited much attention (Tissière & Watson, 1958; Gillchriest & Bock, 1958; Wagman & Trawick, 1958; Bolton, Hoyer & Ritter, 1958; Petermann, Hamilton, Balis, Samarth & Pecora, 1958; Tissière, Watson, Schlessinger & Hollingsworth, 1959; Huxley & Zubay, 1960; Petermann & Hamilton, 1961). In view of these reports it was thought that deoxycholate could be used to prepare ribosomes from rat liver in a form capable of incorporating amino acids into protein *in vitro*, if care were taken to use the optimum concentration of magnesium ions during the preparation. It was soon found that ribosomes could be prepared from rat liver by the normal deoxycholate method, in the absence of magnesium, and that they would incorporate amino acids into protein *in vitro*. The result has been briefly reported (Korner, 1959*a*, 1960*a*), and this paper records the methods used to prepare the ribosomes, reports on their properties and gives the results of some studies carried out with them. Since the earlier

report (Korner, 1959*a*) other workers have obtained amino acid incorporation into protein with ribosomes isolated from mammalian tissues (Takanami, 1960; Rendi & Hultin, 1960; Kirsch, Siekevitz & Palade, 1960) and from *E. coli* (Tissière, Schlessinger & Gros, 1960).

METHODS

Radioactive amino acids. Generally labelled L-leucine (L-[G-^{14}C]leucine), DL-[1-^{14}C]leucine and DL-[1-^{14}C]valine were used and were obtained from The Radiochemical Centre, Amersham, Bucks.

Chemicals. The sodium salts of ATP and GTP were obtained from Sigma Chemical Co. The sodium salt of creatine phosphate was prepared by the method of Ennor & Stocken (1948) and creatine phosphokinase was prepared by the method of Kuby, Noda & Lardy (1954).

Preparation of microsomes and ribosomes. Young adult male and female albino rats were used. They were starved overnight to deplete the liver of most of its glycogen and were killed by decapitation. The liver was removed as rapidly as possible into ice-cold 0·44 M-sucrose and was cut into small pieces with scissors. The liver was blotted with filter paper, weighed by displacement in fresh, ice-cold 0·44 M-sucrose and homogenized in 5 vol. of ice-cold 0·44 M-sucrose in a hand-operated homogenizer of the type described by Dounce, Witter, Monty, Pate & Cottone (1955), which was kept cold in an ice bath. Only three strokes (up and down) of the plunger were used since severe damage to the tissues diminishes its capacity to incorporate amino acids into protein *in vitro* (Zamecnik & Keller, 1954). Mitochondria, nuclei and cell debris were removed from this brei by centrifuging for 10 min. at 15 000*g* at 0° in a refrigerated centrifuge, and most of the supernatant fluid was carefully removed.

Two techniques were used for the preparation of ribosomes from this supernatant fluid. The first was based on that described by Littlefield *et al.* (1955), which was, in its turn, based on the observations of Palade & Siekevitz (1956) and Strittmatter & Ball (1952). The microsomes were separated from the soluble fraction of the cell by centrifuging at 105 000*g* for 1 hr. at 0° in a Spinco model L preparative ultracentrifuge. The soluble fraction of the cell was removed from the pellets, which were rinsed with sucrose, and the walls of the tubes were dried with tissue paper. Half of the microsomal pellets obtained were used directly for incorporation experiments, and the rest were used to prepare the ribosomes by suspending them by gentle homogenization in a freshly made, ice-cold 5% solution of sodium deoxycholate in 0·2 M-glycylglycine buffer at pH 8·2. The volume of the sodium deoxycholate solution added was that which contained about 0·75 mg. of deoxycholate/estimated mg. of microsomal protein. After standing in ice for 10 min., ice-cold water (or, in some experiments, ice-cold 0·03 M-tris buffer at pH 8·2 containing 5 mM-$MgCl_2$) was added to give a final concentration of deoxycholate of 0·5%, and the ribosomes were separated from the solubilized lipoprotein of the endoplasmic reticulum by centrifuging at 105 000*g* for 2 hr.

The second method used was to treat the supernatant fluid after the centrifuging at 15 000*g* with one-ninth of its volume of an ice-cold, freshly prepared 5% sodium deoxycholate solution in 0·03 M-tris buffer at pH 8·2 and to separate the ribosomes by centrifuging at 105 000*g* for 2 hr. (Hall & Doty, 1958). At this stage in both methods, the supernatant was removed, the tubes were drained carefully of as much of the supernatant as possible without loss of pellet material, and the walls wiped with tissue paper. In the earlier experiments the pellet was often loose, which made it difficult to remove all the supernatant fluid, but later it was found that if the rats were starved overnight the pellets obtained were more tightly packed, possibly because of the absence of liver glycogen, which spins down with the ribosomes.

It soon became apparent that the particles could be washed with buffer without loss of incorporating ability, and such a wash became part of the preparative routine. The pellets were suspended by gentle homogenization in as large a volume as possible (usually 12·5 ml./pellet) of 0·03 M-tris buffer at pH 7·5 containing 5 mM-$MgCl_2$, 0·08 M-KCl and 0·05 M-NaCl and were sedimented again by centrifuging, and the washings carefully removed.

Conditions of incubation. The pellets were finally suspended by gentle homogenization in ice-cold 0·045 M-tris buffer at pH 7·5 containing 7·5 mM-$MgCl_2$, 0·12 M-KCl and 0·075 M-NaCl. Samples (0·5 ml.) of the suspended ribosomes were pipetted into ice-cold 15 ml. centrifuge tubes containing 0·25 ml. of the cell sap in 0·44 M-sucrose, or 0·25 ml. of a preparation of pH 5 enzymes (Hoagland *et al.* 1956) in 0·44 M-sucrose, or 0·25 ml. of 0·44 M-sucrose alone. To this was added 0·25 ml. of 0·03 M-tris buffer at pH 7·5 containing 5 mM-$MgCl_2$, 0·08 M-KCl and 0·05 M-NaCl, in which was dissolved ATP, GTP, creatine phosphate, creatine phosphokinase, radioactive amino acid and, in some experiments, glutathione, in concentration to give 1 mM-ATP, 0·02 M-creatine phosphate, 0·6 mM-GTP, 2·5 mM-glutathione, 0·03 mg. of creatine phosphokinase and 0·5 μC of radioactive amino acid in the 1 ml. final vol. of incubation mixture. The tubes were shaken for various lengths of time at 37° in a Dubnoff bath. In most experiments penicillin (100 units) and chloramphenicol (40 μg.) were added to the incubation mixture. The reaction was stopped by the addition of 0·5 N-$HClO_4$ or, if the subcellular particles were to be reseparated from the incubation mixture, by adding ice-cold 0·44 M-sucrose containing an excess of unlabelled amino acid, corresponding to the radioactive amino acid used, and centrifuging at 105 000*g* for 2 hr. at 0°. The microsomes or ribosomes were then resuspended in water and their proteins and those of the soluble fraction precipitated with 0·5 N-$HClO_4$.

Preparation of radioactive proteins. The precipitated proteins were repeatedly washed with 0·5 N-$HClO_4$ containing unlabelled amino acid to remove radioactive amino acids, RNA and lipids were extracted, and the residual protein was dried, plated on weighed aluminium planchets and counted in a Geiger–Müller gas-flow counter as described by Korner (1959*b*). Sufficient counts were recorded to reduce the standard error to 3% or less, and corrections were applied for the background counts. The results are given as counts/min./mg. of protein or counts/min./mg. of RNA.

Determination of ribonucleic acid. RNA was determined on samples of microsomes and ribosomes by the adaptation of the method of Scott, Fraccastoro & Taft (1956) described by Korner (1959*b*). The protein content of the residue after extraction of the RNA was estimated and the RNA:protein ratio calculated.

Determination of nitrogen. The N content of samples of the microsomes and ribosomes, cell sap, pH 5 enzymes and of the residual protein left after the RNA had been extracted in the samples used for RNA determination was determined by the micro-Kjeldahl technique. Corrections were applied for the N content of any tris buffer and of any RNA present, and the corrected value of N was used in calculation of the protein content of the samples by using the factor 6·25.

Measurements in the analytical ultracentrifuge. Samples of the ribosomes and of the microsomes were examined during centrifuging at 37 020 rev./min. or at lower speeds in the Spinco model E analytical ultracentrifuge in 0·03 M-tris buffer at pH 7·5 containing various concentrations of Mg^{2+} ions. The sedimentation coefficients (S) were calculated and were corrected to water and 20°.

The relative proportions of the particles with different S values was obtained by estimation of the area of each peak of the schlieren diagram. This estimation was done in two ways: by counting squares on enlarged photographs of the peaks or by weighing the peaks cut out from the enlarged photographs.

Determination of amino acids. The free amino acid content of the fractions was determined by a modification of the ninhydrin method of Moore & Stein (1948) which has been described by Korner (1959*b*).

RESULTS

The ribosomes prepared by either of the methods described are a yellowish straw colour in suspension possibly because of contamination by ferritin, which can be removed by precipitating the ribosomes with barium (Hamilton & Petermann, 1959; Petermann & Hamilton, 1961). That the preparations are essentially free of contaminating microsomes and endoplasmic reticulum is indicated by the high RNA:protein ratio (mean about 0·8:1 ranging from 0·65:1 to 0·9:1) and by the observation that a second treatment with 0·5% deoxycholate caused little alteration in the RNA:protein ratio of the ribosomes and in their amino acid-incorporating ability.

The sedimentation patterns obtained with schlieren optics when the ribosomes were centrifuged in the analytical ultracentrifuge and the effect of various concentrations of magnesium ions on these patterns were broadly similar to those obtained by other workers using ribosomes from liver and other tissues (Hamilton & Petermann, 1959; Petermann & Hamilton, 1961). Thus peaks with approximate S values of 45, 55, 60, 80, 110 and 140s were noted and the addition of higher magnesium ion concentrations resulted in more of the ribosome preparation sedimenting with higher S values.

Comparison of amino acid incorporation into ribosomes and into microsomes. Ribosomes have been prepared many times by both the methods described, and on each occasion they have shown active incorporation of amino acids into protein when incubated *in vitro* under suitable conditions. Deoxycholate is inhibitory to the system and in order to achieve high rates of incorporation it is important to remove as much as possible of the deoxycholate before suspending the pellet in the incubation medium. Ribosomes can be washed by suspension in the tris buffer–salts solution followed by centrifuging without loss of incorporating activity, and, indeed, with some enhancement of activity probably because of the removal of last traces of deoxycholate. Table 1 records some data on the incorporation shown by microsomes and by washed and unwashed ribosomes during a 60 min. incubation experiment.

Ribosomes incorporate more amino acid/mg. of protein than microsomes. Washed ribosomes show a slightly greater specific activity than unwashed microsomes even when this is calculated per milligram of RNA incubated, which is a fairer method of comparing the activities of the ribosomes and microsomes. In earlier experiments (Korner, 1959*a*), before it was realized that it was important to remove as much as possible of the deoxycholate during preparation of the ribosomes even at the expense of losing some of them, the incorporation found per milligram of RNA was less in ribosomes than in microsomes. This position is reversed when washed particles are used, especially if long incorporation times are employed since ribosomes continue to incorporate for longer periods than microsomes (see below).

The radioactivity of the ribosome protein is not washed out by reincubation of the labelled ribosomes in the presence of excess of unlabelled amino acid.

Washed ribosomes resemble microsomes in requiring the addition of cell sap before full incorporation occurs and the whole cell sap can be replaced by a pH 5 enzyme preparation (Table 2). Boiled cell sap is no substitute for the fresh material. Table 2 shows that full incorporation

Table 1. *Comparison of the incorporation of* DL-[1-^{14}C]*leucine into the protein of microsomes and of washed and unwashed ribosomes*

Incubation was carried out for 60 min. as described in the Methods section. Washing was by resuspension in the tris-buffered salts medium followed by centrifuging.

	Counts/min./mg. of microsomal or ribosomal protein	Counts/min. incorporated into protein/mg. of microsomal or ribosomal RNA incubated
Microsomes	214	960
Ribosomes	632	840
Washed ribosomes	848	1085

Table 2. *Requirements for incorporation of* DL-[1-^{14}C] *leucine into the protein of washed ribosomes*

The complete system is described in the Methods section. Incubation was carried out for 2 hr.

	Counts/min./mg. of ribosomal protein
Complete system	716
Soluble fraction omitted	67
pH 5 Enzyme in place of soluble fraction	655
Boiled soluble fraction in place of soluble fraction	89
GTP omitted	489
ATP omitted	309
ATP-generating system omitted	289
ATP and ATP-generating system omitted	92
Ribonuclease added (0·5 mg./ml.)	48
Deoxycholate added (0·5%)	19
(0·1%)	142
Puromycin added (2 μM)	472
(20 μM)	101
(600 μM)	19

requires the addition of GTP and ATP but that the washed ribosomes show less dependence on an ATP-generating system than microsomes (Zamecnik & Keller, 1954) or unwashed ribosomes (Korner, 1959*a*). Further, it is possible to obtain high levels of incorporation when the ATP-generating system is omitted provided that higher ATP concentrations than the usual 1 mM are used. This point is discussed below.

Puromycin at a concentration of 10 μM caused 80% inhibition of incorporation (cf. Yarmolinsky & de la Haba, 1959) and 98% inhibition was obtained with a concentration of 600 μM. The presence of ribonuclease is, as was expected, inhibitory to the system and so is the presence of deoxycholate (Table 2).

Time course of labelling. Fig. 1 (*a*) compares the increase in specific activity with time of incubation of the proteins of microsomes and of washed ribosomes incubated under identical conditions. The microsomes incorporate amino acids into protein for about 30 min. and the specific activity subsequent to this time remains at a plateau (cf. Hoagland *et al.* 1956; Zamecnik & Keller, 1954), but the specific activity of ribosome protein continues to rise for 2 or 3 hr. Ribosomes prepared without the precautions designed to remove all the deoxycholate stop incorporating much earlier (Korner, 1959*a*). If the results depicted in Fig. 1 (*a*) are expressed as counts/min./mg. of RNA instead of counts/min./mg. of protein then it becomes clear (Fig. 1*b*) that microsomes are more rapidly labelled with radioactive amino acids than ribosomes but that they stop incorporating amino acids very much earlier than ribosomes.

During the incubation procedure labelled protein appeared in the supernatant fluid. A typical experiment is illustrated in Table 3, which shows than when ribosomes are incubated with cell sap or with a pH 5 enzyme preparation, the protein of the supernatant fluid became progressively labelled with amino acid until about 20% or more of the total counts were present in non-ribosomal protein. That the incorporation of amino acids into ribosomal protein and into the supernatant fluid

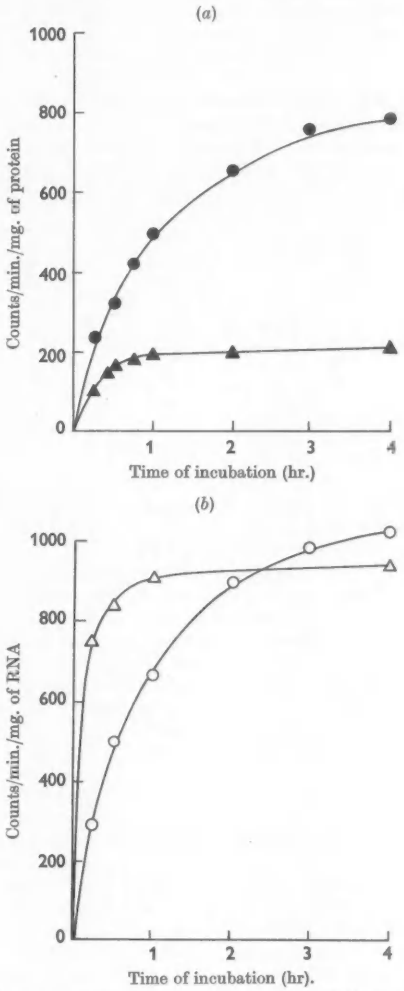

Fig. 1. Time course of incorporation of L-[G-^{14}C]leucine into protein of ribosomes (●, ○) and microsomes (▲, △). (*a*), Expressed as counts/min./mg. of protein; (*b*), expressed as counts/min./mg. of RNA.

Table 3. *Appearance of radioactive proteins in the supernatant fluid during incubation of ribosomes (1·6 mg. of protein) with* DL-[1-^{14}C]*leucine in the presence of either cell sap (2·2 mg. of protein) or a* pH 5 *enzyme preparation (2·0 mg. of protein)*

Incubation time (min.)	Incubation with cell sap: Total counts in Ribosome protein	Incubation with cell sap: Total counts in Cell-sap protein	Incubation with cell sap: Counts in cell-sap protein (% of total)	Incubation with pH 5 enzymes: Total counts in Ribosome protein	Incubation with pH 5 enzymes: Total counts in pH 5 enzyme protein	Incubation with pH 5 enzymes: Counts in pH 5 enzyme protein (% of total)
0	12	4·5	—	28	2	—
30	291	42	12·6	167	29	14·8
60	570	129	18·4	311	84	21·3
120	795	198	19·8	520	157	23·2

Table 4. *Effect of preincubation of ribosomes and microsomes at 37° on their subsequent ability to incorporate* DL-[1-^{14}C]*leucine into protein* in vitro

Preincubation was carried out in the tris-buffered medium containing salts described in the Methods section.

Time of preincubation at 37° (min.)	Counts/min./mg. Ribosome protein	Counts/min./mg. Microsome protein
0	525	298
15	537	141
30	502	79
45	487	47
60	442	36
Ribosomes prepared from microsomes which had been preincubated for 30 min.	168	

protein was larger in the experiment in which cell sap was used than in the one when pH 5 enzyme was used is probably not significant.

All experiments in which long incubation times were used were carried out in the presence of 100 units of penicillin and 40 μg. of chloramphenicol/1 ml. of incubation mixture, and the absence of serious bacterial contamination was noted when samples of the incubation mixture after incubation were plated on a tryptic digest of casein supplemented with Marmite and glucose.

Stability of ribosomes. That incorporation of amino acids into protein occurs in ribosomes for longer periods of time than in microsomes argues that ribosomes are much more stable than microsomes. Preincubation experiments confirm this suggestion. Ribosomes, or microsomes, suspended in the buffered tris–salts medium were shaken at 37° for various periods before adding the cell sap, ATP, ATP-generating system, GTP and radioactive amino acid. The mixture was then incubated for 2 hr. and the results are shown in Table 4. Microsomes are extremely sensitive to exposure to 37° and a big fall in incorporating ability is found within 15 min. of preincubation, but the ribosomes show little decrease in incorporating ability until after an hour of preincubation. Ribosomes prepared from preincubated microsomes show diminished ability to incorporate amino acids into protein.

Washed ribosomes can be stored at 0° (in tubes standing in crushed ice in a vacuum flask kept in the refrigerator) for at least a week without substantial fall in their ability to incorporate amino acids into protein (cf. Kirsch *et al.* 1960).

Freezing the ribosomes in acetone–carbon dioxide and then thawing them resulted in a variable fall in their ability to incorporate amino acids into protein which ranged from 10 to 40 % of that of the unfrozen ribosomes. It appeared that rapid freezing is less damaging than slow freezing and some further loss in incorporating ability occurs on storage at $-15°$. Addition of the soluble fraction before freezing does not, to any substantial extent, protect the ribosomes against the damage caused by freezing, although it has been reported that microsomes are protected by this means (Sachs, 1957). Ribosomes can be dialysed in the cold for 24 hr. with variable (5–30 %) loss of activity.

Effect of magnesium ion concentration on the S values of ribosomes and on amino acid incorporation into protein of ribosomes. Washed rat-liver ribosomes were suspended in 0·03 M-tris buffer, pH 7·5, and various concentrations of magnesium acetate added to separate samples of it, which were then examined in the analytical ultracentrifuge. The *S* values of the peaks in the schlieren diagrams were determined and the area under each peak was estimated. As expected (Hamilton & Petermann, 1959; Petermann *et al.* 1958; Petermann & Hamilton, 1961), more of the larger sizes of ribosomes were noted in the presence of high concentrations of magnesium ions and when no magnesium was added more of the smaller sizes and less of the larger sizes of ribosomes were apparent. Ribosomes bind considerable amounts of magnesium (Petermann & Hamilton, 1961) so that, even when no magnesium had been added to the suspending medium, some was still present.

The extent of amino acid incorporation into ribosomes under the usual conditions but at different magnesium ion concentrations was determined and the results (Fig. 2) showed a marked dependence on the concentration of this ion with a sharp optimum at an added concentration of 5 mM. The experiment depicted in Fig. 2 was carried out in the presence of an ATP-generating system and at only one ATP concentration (1 mM). This magnesium ion concentration is one at which the largest proportion of the ribosomes appeared in the 80s form. It is tempting to use this rough correlation between extent of incorporation and 80s form to suggest that this is the incorporating size of ribosome but several factors counsel against the acceptance of this facile assumption (see Discussion).

Concentration of adenosine triphosphate and amino acid incorporation into ribosomes. As mentioned above, the ATP-generating system is not obligatory for amino acid incorporation into ribosomes as it is for incorporation into microsomes. Fig. 3 shows the results of incorporation experiments in which the creatine phosphate and creatine phosphokinase were omitted and in which various concentrations of ATP containing equimolar magnesium chloride were present. The incubation mixture had in addition the usual amount of magnesium (5 mM). Incorporation at a rate which was at least as high as that obtained with the complete incubation mixture could be obtained in the absence of an ATP-generating system provided that about five times the usual ATP concentration (plus equimolar magnesium) was used (i.e. 5 mM instead of 1 mM). This concentration of ATP appeared to be optimum, higher concentrations causing inhibition of incorporation.

However, the excess of ATP might be causing inhibition because the magnesium added with it, together with the magnesium in the incubation mixture, brings about the inhibition (cf. Fig. 2). To test this possibility, incorporation of amino acids into ribosomes was measured at a variety of ATP concentrations each at various magnesium ion concentrations and the results are shown in Fig. 4. At each magnesium concentration, the amount of ATP that gives the highest incorporation is approximately equimolar and even small deviations either side of equimolarity cause large changes in ability to incorporate amino acids into protein. It is clear that optimum incorporation is obtained with 0·01M-ATP and 0·01M-magnesium ions. Although higher concentrations of ATP (plus equimolar magnesium) are inhibitory, very high concentrations of ATP, which are by themselves almost completely inhibitory (see Fig. 3), can be tolerated to some extent if equimolar magnesium is present.

A series of curves comparable with those of Fig. 4 can be drawn in which incorporation at various magnesium concentrations is plotted, each at a variety of ATP concentrations. They show that ATP can protect to some extent against the inhibition of incorporation caused by high magnesium concentrations (cf. Fig. 2).

Need for other amino acids before incorporation can occur. One of the criteria that must be satisfied before amino acid incorporation in a cell-free system can be regarded as an indication of protein biosynthesis is that the system must be shown to need a supply of amino acids other than the radioactive one. Some systems satisfy this condition, notably the disrupted bacterial system (Gale & Folkes, 1955) the pea-seedling-ribosome system (Webster, 1959) and the mammalian mitochondrial system (McLean, Cohn, Brandt & Simpson, 1958). This requirement has not been met for the liver microsomal system, and it has been assumed that this system contains sufficient free amino acid to supply the materials needed for protein synthesis.

Although boiled cell sap was no substitute for fresh cell sap, addition of boiled cell sap to the complete system resulted in enhanced incorporation of the radioactive amino acid, especially if only

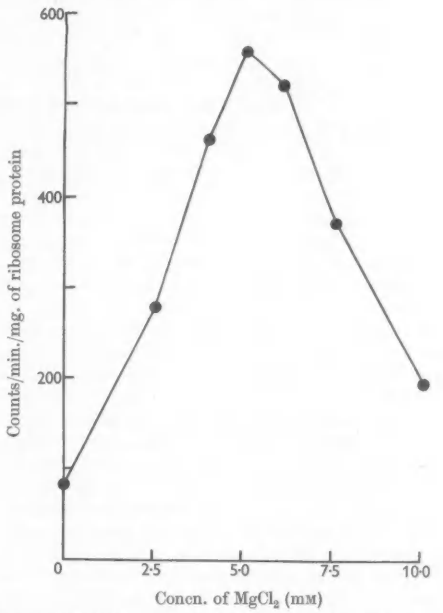

Fig. 2. Effect of concentration of magnesium ions on the incorporation of DL-[1-^{14}C]leucine into ribosome protein.

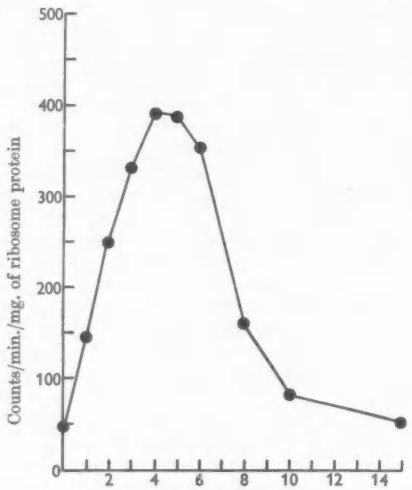

Fig. 3. Effect of concentration of ATP on incorporation of DL-[1-^{14}C]valine into ribosome protein. The complete system (see Methods section) minus the ATP-generating components was used. ATP and an equimolar amount of magnesium chloride were added. Final concentrations shown (see text).

small concentrations of pH 5 enzyme or of fresh cell-sap protein were present. The rise may possibly be caused by the addition of amino acids present in the boiled cell sap to the incubation mixture.

In an attempt to demonstrate more directly the need for amino acids other than the radioactive one in the ribosome system, ribosomes and pH 5 enzyme preparations were dialysed overnight at 4° to deplete them of free amino acids. The incorporation obtained with such systems was about 60–90 % of that of the fresh system and in some experiments addition of an amino acid mixture at a concentration of 0·01–2 mg./ml. and lacking the labelled amino acid did cause some enhancement of incorporation (see Table 5). In other experiments, however, especially when incorporation after dialysis was quite high, little or no enhancement of incorporation was obtained on adding unlabelled amino acids. An estimation of the free amino acid content of the ribosomes and pH 5 enzymes after dialysis revealed that during dialysis the free amino acid content fell and then rose again. Presumably as amino acids are dialysed out of the ribosomes or cell-sap suspensions, protein breaks down to replace the free amino acids.

Attempts were made to free concentrated preparations of ribosomes and pH 5 enzymes from amino acids by treatment with various ion-exchange resins and also with Sephadex (G·25, from Pharmacia, Uppsala, Sweden). After filtering off the resin or Sephadex, the ribosomes and pH 5 enzyme effluents were diluted with buffered tris medium containing salts at concentrations calculated to give the molarities in the final incubation mixture which are usually used. Little incorpora-

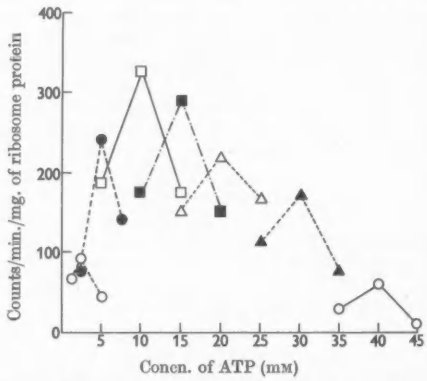

Fig. 4. Effect of concentration of ATP on incorporation of DL-[1-^{14}C]leucine into ribosome protein at different concentrations of magnesium ions. The complete system minus the ATP-generating components was used. Concentrations of magnesium ions (mM): ○--○, 2; ●, 5; □, 10; ■, 15; △, 20; ▲, 30; ○—○, 40.

Table 5. *Effect of adding an amino acid mixture on incorporation of* L-[G-^{14}C]*leucine into ribosome protein in a ribosome–*pH 5 *enzyme system which has been dialysed*

The amino acid mixture consisted of 1 part of histidine, hydroxyproline, cysteine and tryptophan; 2 parts of methionine, arginine, tyrosine, isoleucine, aspartic acid, glycine, alanine, proline, threonine, glutamine and asparagine; 4 parts of lysine, serine, valine and glutamic acid; all in the L-form.

Amount of amino acid mixture added (mg./ml.)	Counts/min./mg. of ribosome protein
0	312
0·01	419
0·1	432
1·0	369
2·5	218
5·0	94
10·0	32

tion of radioactive amino acid occurred after this treatment, and, although some enhancement of incorporation was observed when an amino acid mixture was added, it was clear that inactivation of the ribosomes and pH 5 enzymes had occurred during treatment with the resins, for, even when optimum concentrations of amino acids were added, the incorporation obtained was small compared with that of the untreated systems.

High concentrations of the amino acid mixture (lacking the radioactive amino acid) were inhibitory to the system (see Table 5). The degree of inhibition reached 90% in some experiments, but the amount of amino acid mixture needed to cause inhibition to this extent varied from experiment to experiment in a way that is related to the amount of cell-sap protein or pH 5 enzyme protein used. Inhibition of incorporation of L-[G-^{14}C]-leucine was also obtained with high concentrations of mixtures containing only eight of the amino acids, and in one experiment 1·0 mg. of isoleucine/ml. alone was sufficient to cause 40% inhibition, but a similar concentration of valine did not cause any inhibition of incorporation.

DISCUSSION

The results presented in this paper confirm the earlier reports (Korner, 1959*a*, 1960*a*) that it is possible to prepare particles from rat liver which are identical with the morphological entities, microsomal particles (Palade, 1955, 1958) or ribosomes (Roberts, 1958), and that these particles will incorporate amino acid into protein *in vitro*. Since the earlier report (Korner, 1959*a*), Takanami (1960) has described the preparation of ribosomes from rat liver by precipitation with bivalent cations and the ribosomes were shown to incorporate amino acids into protein. Rendi & Hultin (1960) used deoxycholate containing magnesium to prepare ribosomes from rat liver and Kirsch *et al.* (1960) report the separation of ribosomes from guinea-pig liver. Both of these preparations of ribosomes can incorporate amino acids into protein *in vitro*. In addition, incorporation of amino acids into protein *in vitro* has been obtained with ribosomes from *E. coli* (Tissière *et al.* 1960) and pea seedlings (Webster, 1959).

Preparation of ribosomes. In order to obtain incorporation of amino acids into ribosome protein it is important to remove as much as possible of the deoxycholate from the ribosome pellet before incubation because of the inhibitory action of the detergent. Littlefield *et al.* (1955) appear to have been in the practice of taking up the loose ribosome pellet together with some of the supernatant fluid, and the 0·5% deoxycholate contained in the fluid would probably account for their finding that the particles were inactive (Littlefield & Keller, 1957). These workers were clearly aware of the inhibitory nature of deoxycholate and themselves suggest that this inhibition might account for their inability to find incorporation of amino acids into microsomal particle protein.

The addition of magnesium ions at a concentration of 5 mM to the sucrose used to homogenize the liver, and the presence of this amount of magnesium throughout the course of preparation of ribosomes, did not appear to raise the amount of amino acid incorporated into ribosomes in the subsequent experiments. The yield of ribosomes did, however, appear to be somewhat greater when magnesium was present and this is understandable in view of the fall in the proportion of ribosomes that are of low S values with increasing magnesium ion concentration (Petermann *et al.* 1958; Petermann & Hamilton, 1961). It is to be expected that a little less of the ribosomes would spin down during the preparative procedures in the absence of magnesium.

Stability of ribosomes. Ribosomes are more stable than microsomes. They can be preincubated, stored at 0°, and can be dialysed without substantial loss of incorporating ability, and they continue to incorporate amino acid into protein at 37° for a much longer time than the microsomes from which they are prepared. Kirsch *et al.* (1960) report that guinea-pig-liver ribosomes can be stored at −10° without loss of activity but that they lose most of their activity if stored overnight at 0°. This is opposite to the findings with rat-liver ribosomes reported in this paper.

Requirements for incorporation. Ribosomes are like microsomes in requiring for full incorporation the presence of GTP, ATP and either the cell sap or pH 5 enzymes. They are very sensitive to the presence of the correct concentration of ions and are especially sensitive to the concentration of magnesium. The optimum pH is between 7·3 and 8·0 (cf. Takanami, 1960). Unlike the microsome system, however, an ATP-generating system is not necessary provided extra ATP and magnesium, over and above that usually used in experiments with microsomes and ribosomes, is added. It is possible that adenosine-triphosphatase activity is removed from microsomes along with the lipoprotein of the endoplasmic reticulum when ribosomes are prepared. Takanami (1960), Rendi & Hultin (1960), Kirsch *et al.* (1960) and Tissière *et al.* (1960) all report the need for an ATP-generating system in their various ribosome systems, but in each case they used 1 mM-ATP in the experiments in which an ATP-generating system was omitted. It would be of interest to learn if higher ATP concentrations (together with, of course, the correct amount of magnesium) would show that a ATP-

generating system was not needed in their ribosome systems. Littlefield & Keller (1957) found that ribosomes isolated from ascites-tumour cells did not require an ATP-generating system.

Cohn (1959) reports that addition of cell sap is not necessary for amino acid incorporation into protein of particles prepared from microsomes with a non-ionic detergent, Lubrol-W. He suggests that the amino acid-activating enzymes are attached to microsomes and are made more accessible by the Lubrol treatment. It is further suggested that soluble RNA is not essential as an intermediate between the activated amino acids and the Lubrol or perfluoro-octanoate pellet (Cohn, 1959), or between activated amino acids and microsomes (Rendi & Campbell, 1959). The experiments reported in this paper make it clear that washed ribosomes do require added cell sap and do not have activating enzymes attached to them. The inhibition of incorporation by puromycin suggests that soluble RNA is an obligatory intermediate for incorporation into protein in this system (Yarmolinsky & de la Haba, 1959).

It is disappointing that a complete dependence of incorporation on the presence of a full complement of amino acids could not be demonstrated, but the evidence that was obtained was suggestive of such a dependence. Kirsch *et al.* (1960) report a similar failure to show stimulation of incorporation on adding an amino acid mixture to ribosomes from guinea-pig liver. The inhibition of incorporation by high concentrations of amino acid mixture, and even by one amino acid, is intriguing and is supported by similar observations of Kirsch *et al.* (1960) and Tissière *et al.* (1960).

Transfer of protein from ribosomes. Radioactively labelled protein could be detected in the supernatant fluid during incubation and the supernatant protein became progressively more highly labelled throughout the incubation. It is possible that it arises because breakdown of the ribosomes occurs during incubation, but the stability of ribosomes suggests that the labelled protein is transferred from the ribosomes to the supernatant fluid.

Effect of concentration of adenosine triphosphate and magnesium on incorporation. In the experiment in which various ATP and magnesium concentrations were added, the dual role of magnesium and of ATP becomes clear (Figs. 2–4). With 1 mM-ATP (Fig. 2) concentrations of magnesium below 5 mM are less efficient than the optimum concentration in causing amino acid incorporation, possibly because magnesium is needed for amino acid activation (cf. Kubovitz & Ott, 1944). Above 5 mM-magnesium ions inhibition occurs unless extra ATP is added to use up some of the magnesium. By the same token, in the absence of an ATP-generating system at least 5 mM-ATP is required (Fig. 3), for this concentration was optimum even though what would appear to be inhibitory amounts of magnesium ions were added with it. In this experiment the magnesium concentration was greater than equimolar and so adding concentrations of ATP + magnesium ions greater than 5 mM were inhibitory possibly because of the addition of the extra magnesium. Where the ATP and magnesium concentrations keep pace with one another (Fig. 4), 0·01 M of each is optimum. High ATP and magnesium concentrations can be added in the presence of the other without completely inhibiting incorporation showing the protection each affords against the other. They are, however, both inhibitory at very high concentrations even in the presence of the other for reasons which can be speculated about but are not clear.

S of ribosomes and incorporation. It is generally agreed that the state of aggregation of ribosomes from many sources is dependent on the ionic strength, composition and pH of the suspending medium (Petermann *et al.* 1958; Hamilton & Petermann, 1959; Petermann & Hamilton, 1961). The role of magnesium ion concentration is very marked. In rat-liver ribosomes, 80s particles can be aggregated in high magnesium ion concentration to 110s and larger particles and 80s ribosomes break down on lowering the magnesium concentrations to 60s, 55s, 45s and smaller particles. Somewhat similar results were obtained by measuring *S* values at different magnesium concentrations in experiments described in this paper. It is, however, difficult to say what sizes of ribosome are actually present during incorporation from experiments of this type, since the other components of the incubation mixture, especially the salts, ATP, GTP and soluble RNA, will effect the state of ribosome aggregation. In addition the concentration of ribosomes used, with the attached magnesium ions (Petermann & Hamilton, 1961), is an important variable. It can be calculated from the work of Petermann & Hamilton (1961) that the ribosomes in a 5 mg./ml. suspension will carry enough magnesium with them to produce an approximately 1 mM concentration.

It is for these reasons that care must be taken not to jump to the conclusion that, because at 5 mM-magnesium ion concentration, good amino acid incorporation is obtained and a high proportion of 80s particles are present that the 80s particle is the incorporating form. At this magnesium concentration in the full incubation mixture ultracentrifugal studies of the whole incubation mixture reveal a variety of particles and the change in the proportion of those present is small when the magnesium concentration is altered

sufficiently to give a very big fall in incorporation. It is possible that incorporation is carried out by a small number of particles of a particular size (Tissière *et al.* 1960) the presence of which is masked by other non-incorporating particles of the same *S*.

Identity of the particles. That the particles studied in the experiments described here are indeed ribosomes seems certain from their RNA:protein ratios, their sedimentation characteristics and from the differences in behaviour between them and the microsomes from which they are derived. Their stability and their ability to transfer a fair proportion of labelled protein to the supernatant during incubation suggest that this system may prove a useful one for study of the mechanism of protein biosynthesis in mammalian tissues.

It is of course realized that no proof that ribosomes can synthesize protein *in vitro* has been presented but preliminary studies have demonstrated their ability to incorporate amino acid into a specific protein (Korner, 1960*b*).

SUMMARY

1. Ribosomes with a high ribonucleic acid:protein ratio have been prepared from rat liver by two methods which are described. They have been shown to incorporate radioactive amino acids into protein *in vitro*.

2. Ribosomes incorporate more amino acid into protein (calculated per mg. of protein or per mg. of ribonucleic acid) than microsomes and they continue to incorporate amino acids for 2 or 3 hr.

3. Adenosine triphosphate, guanosine triphosphate and the soluble fraction of the cell (or a pH 5 enzyme preparation) are required for incorporation but an adenosine triphosphate-generating system is not needed.

4. Ribosomes are more stable than microsomes, being able to withstand preincubation at 37° for an hour, dialysis, and storage at 0° for at least a week without loss of activity.

5. Labelled protein is transferred from the ribosome to the supernatant fluid during incubation.

6. The concentrations of magnesium ions and adenosine triphosphate that give maximal incorporation are very critical, but large amounts of either, which in themselves are inhibitory, can be tolerated to some extent if equimolar amounts of the other are present.

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The Chemistry of Xanthine Oxidase

7. THE ANAEROBIC REDUCTION OF XANTHINE OXIDASE STUDIED BY ELECTRON-SPIN RESONANCE AND MAGNETIC SUSCEPTIBILITY*

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The reduction of xanthine oxidase by substrates and other reducing agents is accompanied by complex changes in the visible absorption spectrum. Part of the change occurs rapidly and can be correlated with the overall kinetics of the enzyme-catalysed reaction (Gutfreund & Sturtevant, 1959), whereas part occurs much more slowly and appears to be due to the reduction of 'inactive' xanthine oxidase (Morell, 1952). The stoicheiometry of the optical changes was studied by Totter & Comar (1956). Bray, Malmström & Vänngård (1959) showed that electron-spin-resonance signals arise when the enzyme is treated with xanthine or sodium dithionite, and the present work consists of a quantitative extension of these studies. It is shown that the development of the signals on reduction of the enzyme consists of rapid and slow phases, whose rate and extent can be correlated with the nature and concentration of the substrate or other reducing agent and the composition of the xanthine-oxidase sample. The results provide information on the chemical nature of the reduced forms of the enzyme that may participate in the xanthine-oxidase reaction and in differences between the 'active' and 'inactive' modifications. Electron-spin-resonance and magnetic-susceptibility measurements have provided additional information on the state and function of the iron of the enzyme.

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EXPERIMENTAL

Reagents and buffers. The purity of the substrates, xanthine (from F. Hoffmann–La Roche and Co. Ltd., Basle, Switzerland), purine (from L. Light and Co. Ltd.) and salicylaldehyde, was checked by absorption measurements before and after complete oxidation by xanthine oxidase. The concentration of reduced diphosphopyridine nucleotide (DPNH; from Sigma Chemical Co., U.S.A.) was determined from the absorption at 340 mμ. Sodium dithionite solutions (1–10 mM) were prepared by dissolving the solid in pyrophosphate buffer, while bubbling nitrogen. The solution was immediately drawn into an all-glass micrometer syringe and standardized by titration with approx. 30 μm-moles of riboflavin phosphate (F. Hoffmann–La Roche and Co. Ltd.) in 2 ml. total volume, the end point being detected by the sharp disappearance of fluorescence under an ultraviolet lamp. Nitrogen was bubbled during the titration and a drifting end point, due to reoxidation, was corrected for by extrapolating to zero time. The concentration of riboflavin phosphate was determined from $\epsilon_{450} = 12\,200$ (Whitby, 1953). Molybdenum pentachloride and molybdenum trichloride dibenzoate were obtained from the Climax Molybdenum Co., New York. A solution of potassium octacyanomolybdenum (V) $[K_3Mo(CN)_8]$ was prepared by oxidizing potassium octacyanomolybdenum (IV) $[K_4Mo(CN)_8]$ with ceric sulphate (Weissman & Cohn, 1957) and a solution of oxotrithiocyanatomolybdenum $[MoO(SCN)_3]$ by dissolving $MoCl_5$ in a mixture of hydrochloric acid, acetone and KSCN (Perrin, 1958). Pyrophosphate buffer (0·05M, pH 8·0–8·2) was prepared by adjusting the pH of the tetrasodium salt with HCl.

Xanthine-oxidase solutions. These were prepared by the ammonium sulphate–butanol procedure, followed by calcium phosphate chromatography and in some cases by electrophoresis or low-temperature ethanol fractionation, as outlined by Haddow, de Lamirande, Bergel, Bray &

Gilbert (1958), Bray *et al.* (1959) and Avis, Bergel & Bray (1955). The solutions were dialysed against pyrophosphate buffer before use; where necessary, concentration was achieved by vacuum dialysis against the same buffer, by using a collodion shell (Membranfiltergesellschaft, Göttingen, Germany). The overall purity of the samples was checked roughly in the ultracentrifuge; the main peak accounted for at least 85% of the sedimenting material in all experiments. The total concentration of xanthine oxidase was calculated from $\epsilon_{450} = 70\,000$ (Bray *et al.* 1959).

Measurements of xanthine-oxidase activity. These were carried out at 23·5° under the conditions used by Avis *et al.* (1955). The effect of temperature on the reaction velocity in the range 5–30° was also studied. Q_{10} was found to be 3·0 below 20°, but fell off somewhat above this temperature; the mean value between 20° and 25° was 2·6. Activity measurements on samples at the end of electron-spin-resonance measurements indicated that no inactivation was caused by freezing, by substrates or by dithionite.

Analyses of flavin–adenine dinucleotide, iron and molybdenum. FAD was measured fluorimetrically, and iron with $\alpha\alpha'$-dipyridyl (Avis, Bergel & Bray, 1956*b*); Mo was measured with toluene-3:4-dithiol (Bray *et al.* 1959). The composition of the samples was essentially as reported by Avis *et al.* (1956*b*) and Bergel & Bray (1958).

Electron-spin-resonance measurements. Samples (generally 0·1–0·2 ml. of about 0·1 mM-xanthine oxidase) were placed in silica tubes (3 mm. internal diameter × 100 mm. overall length); the tubes were fitted with aluminium screw caps which pressed Neoprene gaskets on to the ground tops of the tubes, the metal fittings being joined to the silica by means of epoxide cement. Samples were frozen and measured in a Varian V 4500 spectrometer, with or without 100 kcyc./sec. modulation. Most experiments were carried out at 77°K or at about 120°K. When the Varian low-temperature fitting was used, the screw-top sample tubes were immersed completely in liquid nitrogen.

For quantitative measurement of signal intensities, a standard reference solution, consisting of 2 mM-$Cu(NO_3)_2$ in 25 mM-EDTA (disodium salt) (Malmström & Vänngård, 1960), was run at intervals and the settings of the instrument were kept as constant as possible throughout the series of measurements. Intensities were measured as the maximum deflexion of the trace from the base-line for the peak in question, and where necessary were corrected for variations in the sensitivity setting, in the signal intensity of the reference solution and in the diameter of the sample tube, assuming that the signal was proportional to the square of the internal diameter of the tube. To calculate the absolute concentrations of the unpaired electrons responsible for peaks *A* and *B*, the areas under the electron-spin-resonance absorption curves were compared with those from the copper–EDTA complex and factors for converting peak heights into absolute concentrations were deduced, assuming $s = \frac{1}{2}$, as would be expected for Cu(II), Mo(V) and the flavin–adenine dinucleotide semiquinone free radical (FADH). To check the procedure, the integrated areas for the copper standard and for an equimolar solution of $MoCl_5$ in conc. HCl were compared and found to agree almost exactly. Molybdenum solutions were not used for reference purposes owing to their low stability.

Magnetic-susceptibility measurements. The susceptibility balance of Theorell & Ehrenberg (1951), standardized with nickel chloride, was employed at temperatures of 8°, 10° or 20°. (Only the resting enzyme was measured at 20°.) Deoxygenated solutions of the enzyme were measured against buffer, generally 5–10 min. after anaerobic addition of the substrate or other reducing agent to the cold enzyme solution.

Anaerobic techniques. For the electron-spin-resonance measurements, solutions of the enzyme were deoxygenated by allowing them to stand in a flat open dish, in a layer 1–2 mm. deep, in a glove cabinet. Anaerobic conditions were achieved in the cabinet at 5°, by flushing continuously with moist commercial nitrogen. The flushing was started when the enzyme was placed in the cabinet and was continued for about 15 hr. before the tubes were filled. Glass syringes with polythene jets were employed for introducing enzyme and substrate solutions into the electron-spin-resonance tubes inside the cabinet, while this was still being flushed. The contents of the tubes were mixed, the tubes were fitted with the screw caps, removed from the cabinet via an air-lock and cooled in liquid air. For some critical experiments anaerobiosis was further improved by using argon in place of nitrogen and by recirculating the gas in the cabinet through pyrogallol wash-bottles by means of a pump. In one experiment of the type (Fig. 7) the efficiency of anaerobiosis was checked by returning a sample tube to the glove cabinet and thawing, stirring and refreezing the contents under conditions approximating to those used when the tube was filled; thus the sample was given a double exposure to any residual oxygen in the cabinet. Since this procedure had little effect on the signal intensities, it was considered that oxygen had been sufficiently excluded.

For the susceptibility measurements, the enzyme solution was deoxygenated, and, if necessary, mixed with a substrate, in Thunberg tubes filled with argon. The contents were then rapidly transferred to the apparatus, after flushing both the pipette used for the transfer and the tube of the apparatus, with argon.

RESULTS

Electron-spin resonance of Mo(V) *model compounds*

A series of well-characterized Mo(V) compounds were examined to determine *g* values (*g*, spectroscopic splitting factor) and to find out what linewidths and how much hyperfine structure were to be expected at the temperatures at which the xanthine-oxidase measurements were made (Table 1). The height of the hyperfine peaks on the traces was generally only about one-twentieth of that of the main peaks. The *g* value for $K_3Mo(CN)_8$ agreed with that of Weissman & Cohn (1957).

Types of signal from xanthine oxidase

Four signals have been obtained from xanthine-oxidase samples and these are designated *A*, *B*, *C* and *D* (Table 3). Signals *A* and *B* were described by Bray *et al.* (1959). We have not described signals *C* and *D* before; *C* is shown in Fig. 1. Its shape and position were similar to a part of the unidentified

Table 1. *Electron-spin resonance of* Mo(v) *compounds at* 77° K *or* 120° K

Compound	Solvent	g	Shape of main peak	Line width (gauss)	Hyperfine splitting ($cm.^{-1}$)
(*a*) $MoCl_5$	10 N-HCl	1·948±0·005	Asymmetric	Approx. 50	7×10^{-3}
(*b*) $K_3Mo(CN)_8$	N-HCl	1·99±0·01	Symmetrical	26	$4{\cdot}5\times10^{-3}$
(*c*) $MoO(SCN)_3$	Aq. acetone containing HCl and KSCN	1·938±0·003	Asymmetric	22	7×10^{-3}
(*d*) $Mo(C_6H_5{\cdot}CO{\cdot}O)_2Cl_3$	Ethyl acetate	1·95±0·01	Symmetrical	Approx. 50	7×10^{-3}

References: (*a*) Sacconi & Cini (1954); (*b*) Weissman & Cohn (1957); (*c*) Perrin (1958); (*d*) Larson (1960).

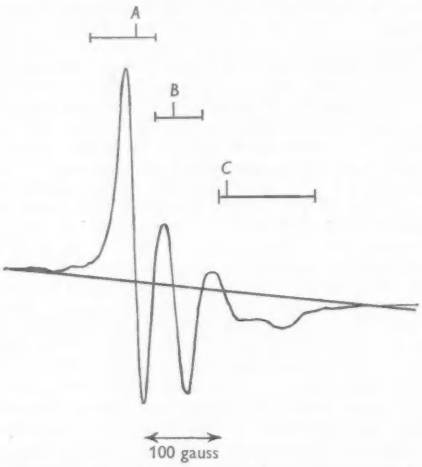

Fig. 1. Electron-spin-resonance absorption curve (first derivative) for a sample of xanthine oxidase reduced with xanthine. Temperature: 77° K; magnetic field increasing from left to right; modulation sweep approx. 12 gauss, peak to peak; the approximate position of the base-line is shown. The curve shows a strong signal *A*, a weaker signal *B* and one of the strongest signals *C* obtained in the present work.

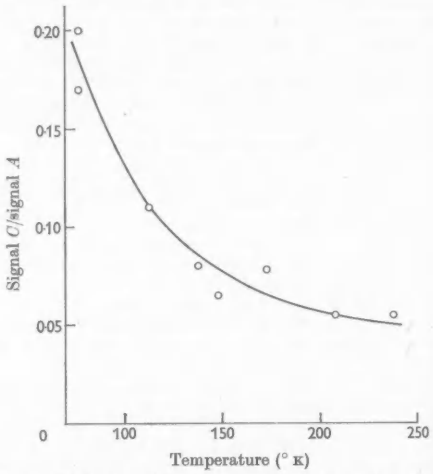

Fig. 2. Variation with temperature of the ratio of the height of signal *C* to the height of signal *A*. The sample of xanthine oxidase (about 0·2 mM) was reduced with xanthine. A constant microwave energy was used.

signal reported by Beinert & Sands (1960; cf. their Fig. 3*e*). On increasing the attenuation of the microwave energy, signal *C* decreased more than did *A* and *B*. Further, whereas signals *A* and *B* could be obtained at either liquid-air or room temperature (Vänngård, Bray, Malmström & Pettersson, 1961), signal *C* almost disappeared on warming samples from 77° K to 230–240° K, but reappeared on cooling (cf. Fig. 2). The signal of Beinert & Sands behaved similarly both with regard to temperature and to microwave-energy variations (Beinert & Sands, 1960; Sands & Beinert, 1960). The intensities of signals *A* and *B* relative to one another were little affected by temperature variations.

The shape of signal *D* was similar to that of the corresponding signal at this g value described by Beinert & Sands (1959).

Conditions affecting intensity of signals. Resting xanthine oxidase did not give signals *A*, *B* or *C*. However, a number of resting samples gave signal *D*. The intensity of this signal varied from one preparation to another and was least on the samples showing the highest purity in the ultracentrifuge. One sample which gave an abnormally intense signal *D* contained approximately 10% of the fast-sedimenting impurity designated *fa* in previous publications (Avis, Bergel, Bray, James & Shooter, 1956*a*); samples which were about 98% pure showed very weak signals. In one solution, which was contaminated with micro-organisms and which might therefore have been partially reduced, signal *D* increased on aeration. Signal *D* remained when the enzyme was reduced anaerobically with xanthine (3 mol.prop.); however, it disappeared on treatment with dithionite (10 mol.prop.).

Signal *C* appeared on anaerobic reduction of xanthine oxidase with xanthine ($\lesssim$ 0·7mol.prop.);

the samples were frozen 2 min. after mixing at 5° and measurements were made at 77° K. The signal was always too weak for precise measurements to be made (Fig. 1). However, all samples examined under suitable conditions have shown the signal, and there was no tendency for purer samples to give less intense signals. The weakness of the signal and the fact that a twofold decrease in its relative intensity took place on changing the temperature from 77° K to 120° K (Fig. 2) explain why signal *C* was not observed by Bray *et al.* (1959). The temperature of measurement reported by these authors as about 100° K was, in fact, probably nearer to 120° K.

The effects on signals *A* and *B* of anaerobic additions of xanthine, purine and dithionite to a number of xanthine-oxidase samples are shown in Figs. 3–7. When samples were frozen 2 min. after the addition of xanthine, signals *A* and *B* were maximal with 1–2 moles of substrate/mole of total xanthine oxidase; with 3 or more moles of substrate, only very weak signals were obtained (Fig. 3*a*). Fig. 5 (*a*) shows the weak signal obtained in a similar experiment with a low-activity sample which had about the same molybdenum content as that in Fig. 3. (The composition of xanthine-oxidase samples is considered under Discussion.) Figs. 3 (*b*, *c*) and 5 (*b*, *c*) show the effect of allowing a longer period for the enzyme and substrate to interact. They indicate that there was a slow phase of the reaction in which both the samples gave signals, particularly with large amounts of substrate. Fig. 4 (*a*–*c*) shows similar curves for the 'active' enzyme sample employed in Fig. 3, but with purine as substrate. Here the maximum was initially in the region of 0·5 mole of purine/mole of enzyme, and the second phase of the

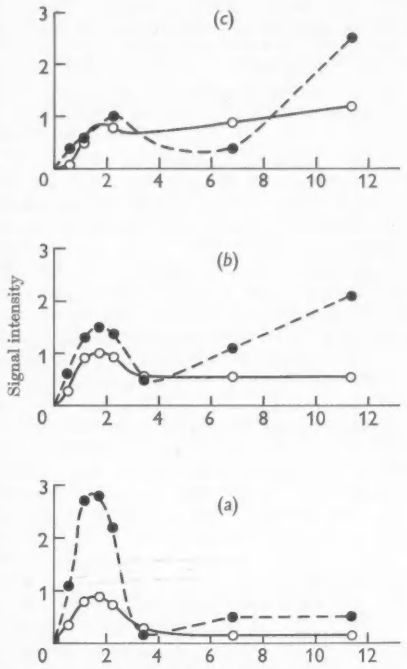

Fig. 3. Anaerobic titration of xanthine oxidase with xanthine. The intensities of signals *A* and *B* in arbitrary units are plotted against the number of moles of xanthine added/mole of total xanthine oxidase. The signal intensity units are roughly the same, and refer to the same total xanthine-oxidase concentration in all of Figs. 3–7; the units for the two signals have been adjusted so that equal ordinates for signals *A* and *B* correspond to equal areas under the integrated absorption curves. The sample of xanthine oxidase used contained 60% of xanthine oxidase-*a*, 15% of xanthine oxidase-i_1 and 25% of xanthine oxidase-i_2 (see Discussion). (*a*) Samples were frozen with liquid air 2 min. after adding the substrate at 4°; each point represents a separate sample. Measurements were made at about 120° K. (*b*) Samples from (*a*) were thawed, incubated for 15 min. at 28°, then refrozen and re-run, without opening the tubes. (*c*) Samples from (*b*) were thawed, incubated for a further 45 min. at 28°, then refrozen and re-run. ○, Signal *A*; ●, signal *B*.

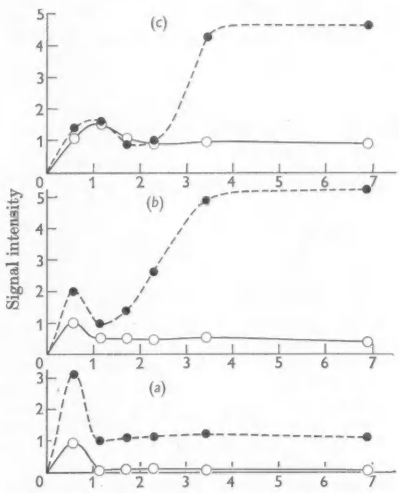

Fig. 4. Anaerobic titration of xanthine oxidase with purine. The enzyme sample and the conditions were the same as those in Fig. 3.

reaction, as far as signal *B* is concerned, was more rapid than with xanthine and had proceeded to a considerable extent even with only 2 min. incubation at 4° (Fig. 4*a*). In other experiments on this same enzyme sample, in which salicylaldehyde was the substrate, the second phase for signal *B* appeared to be more rapid still, so that, even at 2 min., high *B* signals were obtained with all except the smallest amounts of substrate; hence the peak in the curve could only just be distinguished. With DPNH as substrate the behaviour of the 'active' sample was similar to that of the 'inactive' sample with xanthine (Figs. 5*a* and 5*b*), in that there were no peaks in the curves and significant signals were obtained only after incubating for 15 min. at 28°.

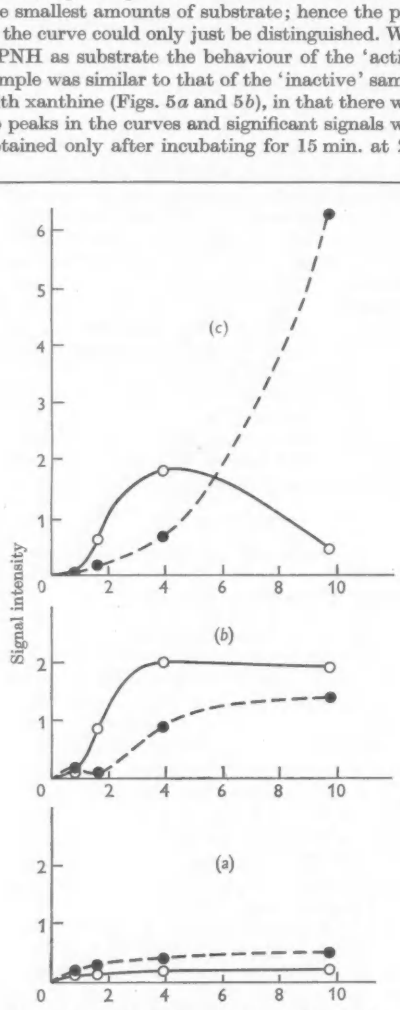

Fig. 5. Anaerobic titration of xanthine oxidase with xanthine. The sample contained 10% of xanthine oxidase-*a*, 70% of xanthine oxidase-i_1 and 20% of xanthine oxidase-i_2; the conditions were the same as those in Fig. 3.

Dithionite-titration curves for two enzyme samples are shown in Figs. 6 (*a*) and 6 (*b*) (cf. Vänngård *et al.* 1961). These curves were obtained by freezing for 2 min. after mixing at about 25°; substantial changes in the intensities, particularly of signal *B*, took place if longer incubation times were employed. In the 'active' sample (Fig. 6*a*) the change consisted mainly of a threefold intensification of signal *B* in the region of 7–9 mol.prop. of dithionite. For the 'inactive' sample (Fig. 6*b*) a similar intensification occurred and the *B* signals

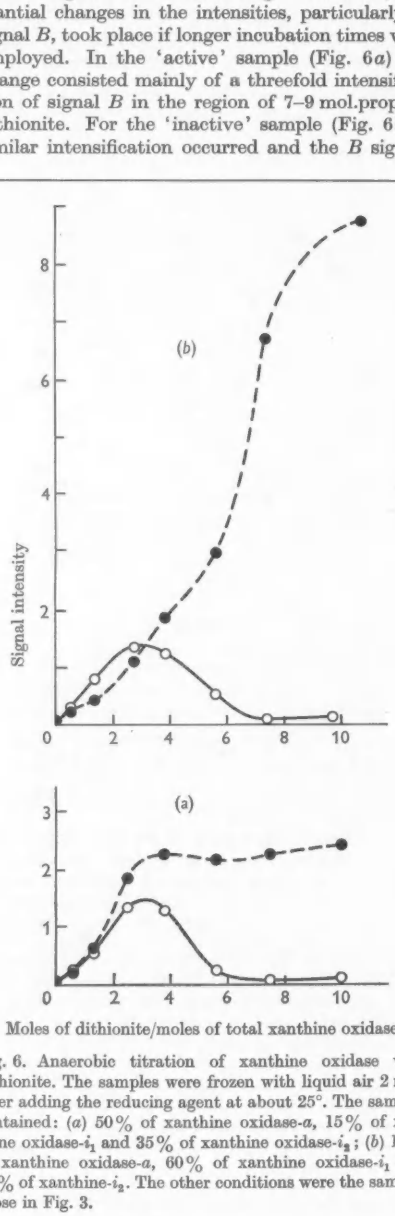

Fig. 6. Anaerobic titration of xanthine oxidase with dithionite. The samples were frozen with liquid air 2 min. after adding the reducing agent at about 25°. The samples contained: (*a*) 50% of xanthine oxidase-*a*, 15% of xanthine oxidase-i_1 and 35% of xanthine oxidase-i_2; (*b*) 10% of xanthine oxidase-*a*, 60% of xanthine oxidase-i_1 and 30% of xanthine-i_2. The other conditions were the same as those in Fig. 3.

obtained under these conditions were in fact the most intense that have been obtained from any xanthine-oxidase samples; the intensification was accompanied by a decrease in intensity of signal *B* in the region of 1–5 mol.prop. of reducing agent. For both the above samples, incubation gave rise to relatively small increases in the intensity of signal *A* in the region of 3 mol.prop. of dithionite. In another experiment, with a sample similar to that used in Fig. 6(*a*), more prolonged incubation at 28° was employed. With 5–7 mol.prop. of dithionite the intensity of signal *B* after 1 hr. or more was the same as that after 15 min. incubation. However, with 10 mol.prop., the signal decreased after its initial rise when the sample was incubated for more than 15 min.

The maximum in the curve obtained after 2 min. with xanthine (Fig. 3*a*) has been investigated in greater detail for a number of enzyme samples. Results obtained under fairly strict anaerobiosis are shown in Fig. 7. In other experiments the positions of the maxima for the two signals always coincided, but the ratios signal *A*:signal *B* were sometimes higher than those shown in Figs. 3(*a*) and 7. However, this may have been due to deterioration of the enzyme, since a number of freshly dialysed samples gave ratios similar to those in Fig. 7. When from a titration series an enzyme–xanthine mixture giving signals near to the peak was thawed, treated anaerobically with more xanthine and refrozen, the signals were markedly diminished. In a somewhat similar experiment, a controlled amount of oxygen (about 2 mol.prop. in the form of buffer saturated with the gas) was added to a sample containing 4 mol.prop. of xanthine. In this case the addition gave an increase in the signal intensities to about the values expected for the peak region.

Absolute intensity of signals. Calculations indicated that in Fig. 3(*a*) the maxima of signals *A* and *B* corresponded respectively to 0·07 and 0·23 equiv. of unpaired electrons/mole of total xanthine oxidase. Similarly, the intense signal *B* obtained on incubating an 'inactive' sample with about 10 mol.prop. of dithionite corresponded to about 1·9 equiv. of unpaired electrons/mole of xanthine oxidase.

Magnetic-susceptibility measurements

Preliminary measurements suggested that the resting enzyme was purely diamagnetic (Bray *et al.* 1959). The more accurate measurements now presented show, however, that the diamagnetism is only about half that of authentic diamagnetic proteins such as the carbon monoxide compounds of myoglobin and ferroperoxidase. This means that the resting enzyme has some paramagnetism; the value given in Table 2 has been calculated by assuming that the diamagnetic contribution is about the same as that of the above proteins, i.e. the volume-susceptibility increment for a 1% solution is $-54(\pm 9) \times 10^{-11}$ c.g.s. units (A. Ehrenberg, unpublished data). This assumption seems justified, since the partial specific volume of xanthine oxidase, $0{\cdot}74 \pm 0{\cdot}02$ (Avis *et al.* 1956*a*), is close to the values for the haemoproteins. The increases in magnetic susceptibility that occurred on treatment with xanthine, purine or dithionite are also shown in Table 2, together with some theoretical values. Measurements were also made on the xanthine-oxidase sample containing 10% of the impurity *fa* which, as stated above, gave a rather high signal *D* in the electron-spin-resonance measurements. The susceptibility increase on

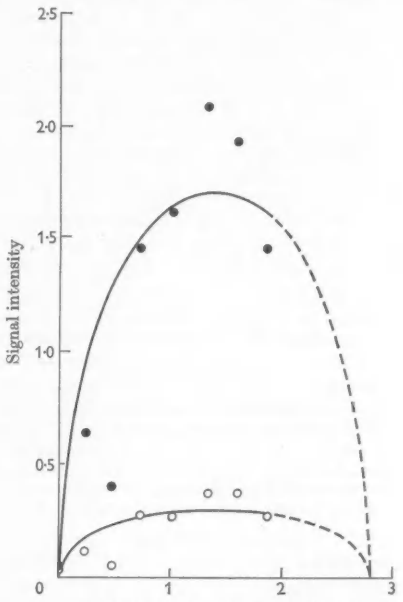

Fig. 7. Anaerobic titration of xanthine oxidase with xanthine. The samples were frozen with liquid air 2 min. after the substrate was added at 5° and measurements were made at 77°K; the preparation contained 50% of xanthine oxidase-*a*. Other conditions were as in Fig. 3. The lines are theoretical curves calculated from the equations of Michaelis & Schubert (1938) for the concentration of an intermediate in a reversible two-step reduction; they were adjusted to obtain the best fit to the data and it was assumed that the concentration of the intermediate did not exceed about 20% of that of the starting material, since in these circumstances the form is independent of the height of the maximum.

adding purine to this sample was only about one-third of the one in the Table. A rather high paramagnetism of the resting enzyme was also indicated, with χ(XO) in the region of 20×10^{-3} units, but this result needs confirmation, since the solution also contained some polyethylene glycol for which no correction could be made.

DISCUSSION

Molecular species responsible for signals

The general conclusions are summarized in Table 3.

It was concluded by Bray *et al.* (1959) and Vänngård *et al.* (1961) that signal *A* was due to the FAD semiquinone free radical (FADH) and signal *B* to Mo(v) or Mo(III). The present experiments have shown: (1) that under certain conditions (e.g. Fig. 3*a*) signal *B* disappears with excess of reducing agent and (2) that 'inactive' samples can give signal *B*. Thus some of the reasoning on which the identification of the signals was originally based has now become invalid. Nevertheless, there seems little doubt that the identification, which now rests primarily on the properties of the signals themselves (cf. Table 3), is correct. The fact that under conditions such as those in Fig. 3 (*a*) the species responsible for signal *B* can apparently be further reduced, so that the signal disappears, suggests very strongly that it is due to Mo(v) rather than Mo(III). In general, rather vigorous conditions are required to reduce molybdenum compounds below the tervalent level. The possibility that in some cases the rather complex signal *B* is due to Mo(v) and in others to Mo(III) is unlikely. It was shown by Bray *et al.* (1959) that, on decreasing the modulation field, the signal became resolved into four or more components, and during the present work this was confirmed. Although variations in the relative intensities of the components were occasionally noted, they were not apparent in those samples from the experiment in Fig. 3 which were examined at low modulation. Hence the same signal-giving species must have been involved throughout. The data on the Mo(v) model compounds (Table 1) are entirely consistent with the assumption that signal *B* is due to Mo(v).

Signal *C* cannot at present be identified with certainty, but the resemblance between it and the '*g*' signal of Beinert & Sands (1960) is striking and it seems probable that they are identical. These workers concluded that their signal probably appeared when iron was reduced. The appearance of signal *C* seems likewise to be associated with a reduction of iron (see last section of Discussion). The temperature-sensitivity of the signal (Bowers & Owen, 1955) lends some support to the hypothesis that *C* is due to resonance absorption by Fe(II) itself. However, as pointed out by Beinert & Sands (1960), no analogies to simple iron complexes

Table 2. *Molar magnetic susceptibility of xanthine oxidase under various conditions and possible contributions of the constituents*

The figure for the resting enzyme is the average of results on four preparations which contained 1·5–4% of *fa*; those for the xanthine-reduced enzyme are averages for two samples which contained 2 and 4% of *fa* respectively; the remaining figures refer to a preparation containing 4% of *fa*. All the preparations used contained about 60% of xanthine oxidase-*a*. The theoretical values (cf. Selwood, 1956) are based on 2FAD, 1·5Mo and 8Fe/mol. of xanthine oxidase (XO), assuming that all of each of the constituents considered is present in the stated form or reacts according to the equations. Susceptibilities are given in milli-c.g.s. units and the errors are estimated possible deviations. For conditions, see the Experimental section.

Experimental	Theoretical
Resting enzyme: χ(XO) = 8±4	Possible contribution to susceptibility of resting enzyme
Increase on reduction	Fe(III), low-spin: 10–20
χ(XO + xanthine) (1·5 mol.prop.) − χ(XO) = 6±2	Fe(II), high-spin: 80
χ(XO + xanthine) (3 mol.prop.) − χ(XO) = 9±2	Fe(III), high-spin: 120
χ(XO + purine) (8 mol.prop.) − χ(XO) = 11±3	Possible increase on reduction
χ(XO + purine) (8 mol.prop.); reoxidized − χ(XO) = 1±2	FAD→FADH: 2·6
χ(XO + dithionite) (7 mol.prop.) − χ(XO) = 15·5±2	Mo(VI)→Mo(V): 2·0
χ(XO + dithionite) (20 mol.prop.) − χ(XO) = 14·5±2	Mo(VI)→Mo(III): 10
	Fe(III), low-spin→Fe(II), high-spin: 65

Table 3. *Electron-spin-resonance signals from xanthine oxidase*

Signal	*g*	Probable source	Remarks
A	2·00	FADH	Cf. Bray, Malmström & Vänngård (1959)
B	1·97	Mo(v)	
C	Approx. 1·9	Fe(II) (high-spin)?	See Fig. 1; cf. Beinert & Sands (1960)
D	4·2±0·1	Fe(III) (high-spin; impurity)	Cf. Beinert & Sands (1959)

have so far been reported. Hence other alternatives, such as the reduction of part of the iron of an enzyme molecule interfering with the remaining tervalent iron, so that this gives absorption *C*, should perhaps also be considered.

There is little doubt that signal *D* is due to the high-spin form of Fe(III). This is consistent with the *g* value and its similarity to Beinert's ferric signals (Beinert & Sands, 1959, 1960). However, it seems clear that the signal is associated not with the enzyme but with an impurity. This impurity may, in fact, be the coloured, fast-sedimenting component described as *fa* by Avis *et al.* (1956*a*).

Forms of xanthine oxidase

Previous work (Avis *et al.* 1956*b*; Bergel & Bray, 1958) has indicated that xanthine oxidase as normally prepared is contaminated with several 'inactive' forms. These may represent degradation products or possibly incompletely formed xanthine-oxidase molecules; they cannot at present be separated from the 'active' form or from one another, nor can they be distinguished spectrophotometrically. The existence of the different forms has to be assumed to account for variations in the specific activity and molybdenum content. If certain simplifying assumptions are made the relative proportions of the species may be calculated, and values so obtained are used below to assist in the interpretation of electron-spin-resonance experiments. The fact that a consistent picture emerges indicates the usefulness of electron-spin resonance for problems of this kind and strengthens the evidence for the existence of the different form of xanthine oxidase.

Since both the specific activity (activity/E_{450}) and the molybdenum content vary, and since there is not a direct correlation between these variations, it is necessary to assume the presence of at least three forms of the enzyme (Table 4). In the present discussion, the possible existence of forms containing only one molybdenum atom/molecule is ignored, as is the possible presence of the inhibitor described by Bray (1959). For the forms in Table 4, it is obvious that the ratio of activity to molybdenum will be maximal for preparations devoid of the molybdenum-containing 'inactive' form, xanthine oxidase-i_1. Previous publications and unpublished results of Bergel & Bray indicate that this ratio does in fact tend towards a maximum: the highest value obtained was for a crystallized sample for which activity/E_{450} was 82 and Mo/FAD was 0·70, corresponding to an average molybdenum content of 1·40 g.atoms/mole. To deduce the activity/E_{450} value of pure xanthine oxidase-*a*, it is assumed that the above sample was completely devoid of xanthine oxidase-i_1. The activity/E_{450} value is then obtained by extrapolating to 2 atoms of molybdenum/enzyme molecule and is thus $82 \times 2/1{\cdot}40$ (= 117). In fact, samples having activity/E_{450} values of about 100 have been obtained on several occasions (Avis *et al.* 1955) and values in the region of 110 have been found by D. A. Gilbert & F. Bergel (unpublished experiments). Unfortunately no samples with such high activities have yet been analysed for molybdenum: the highest recorded Mo/FAD value is 0·87 (Avis *et al.* 1956*b*). Independent justification for pure xanthine oxidase-*a* having activity/E_{450} of about 117 is provided by the work of Morell (1952) and of Gutfreund & Sturtevant (1959), who calculated the turnover number of the pure active form by an entirely different type of extrapolation based on changes in the 450 mμ absorption band which occurred after anaerobic addition of substrate to the enzyme. Morell obtained a value of 313 min.$^{-1}$/mole of active FAD at 19° and Gutfreund & Sturtevant one of 8 ($\pm$0·5) sec.$^{-1}$ at 25°. These data may be converted into our activity/E_{450} values by taking ϵ_{450} as 70 000 for xanthine oxidase, $\Delta\epsilon$ as 9600 (Avis *et al.* 1956*b*) for the conversion of xanthine into uric acid, and Q_{10} as 2·6 and introducing a factor of 2 since there are 2 FAD/mol. The resulting activity/E_{450} values are respectively 132 and 114 ($\pm$7), and are thus in good agreement with our own extrapolated value.

The percentages of the various forms are then given by: xanthine oxidase-*a*: 100(activity/E_{450})/117; xanthine oxidase-i_1: 100[(g.atoms of Mo/moles of FAD) − (activity/E_{450})/117]; xanthine oxidase-i_2: 100[1 − (g.atoms of Mo/moles of FAD)]. On this basis the concentration of xanthine oxidase-i_1 is a measure of the excess of molybdenum over activity, and the concentration of xanthine oxidase-i_2 measures the excess of flavin over molybdenum.

Substrate-induced signal changes not related to enzymic activity

Only those changes in the signals that occur rapidly with xanthine, purine and salicylaldehyde can possibly be related to changes in the enzyme essential for its activity. On this basis, all changes that are not complete in the shortest interval between mixing enzyme with substrate and stopping the reaction by freezing must belong to a slow phase of the reaction, which is clearly irrelevant to

Table 4. *Forms of xanthine oxidase assumed to be present*

Form	Fe:FAD:Mo*	Activity/E_{450}
Xanthine oxidase-*a*	8:2:2	117
Xanthine oxidase-i_1	8:2:2	0
Xanthine oxidase-i_2	8:2:0	0

* g.atoms or moles/mole of enzyme.

the catalytic activity. The shortest time interval used in the present work (2 min. at 4°) is of the order of 100 times as long as the time required for the enzyme to be reduced by the substrate (Gutfreund & Sturtevant, 1959). The velocity of the secondary changes in the intensity of signals *A* and *B* (Figs. 3–5) is of the same order of magnitude as the slow phase in the reduction of the enzyme observed by Morell (1952). It seems probable that both phenomena are related to the reduction of 'inactive' xanthine-oxidase molecules, as was originally suggested by Morell. This view is strengthened by the fact that secondary signal changes were larger in a sample that contained excessive amounts of the 'inactive' xanthine oxidase-i_1 (Fig. 5) than in one which contained relatively little of this species (Fig. 3).

The velocity of the secondary changes in signal *B* depended on the amount of substrate present and increased in the order xanthine, purine, salicylaldehyde. This would be expected if the substrate with the lowest redox potential (Anderson & Plaut, 1949) gave the most rapid reduction. It follows that the reduction of xanthine oxidase-i_1 (or at least of the molybdenum of this species) proceeds by direct interaction with the substrate. On the other hand, the velocity of the secondary changes in signal *A* were independent of the substrate used and of its concentration (Figs. 3 and 4). Hence it seems that reduction of 'inactive' flavin proceeds predominantly by some secondary process which is not substrate-dependent. Thus, the suggestion of Morell (1952), that the slow reduction of 'inactive' enzyme is due to interaction with the reduced 'active' forms, is probably correct for xanthine oxidase-i_2 but incorrect for xanthine oxidase-i_1.

Substrate-induced signal changes probably related to enzymic activity

From the argument presented above only the peak in the signal *A* and signal *B* curves can possibly be related to the enzymic activity. Peaks were observed with three substrates known to be oxidized rapidly and for a number of preparations containing substantial proportions of xanthine oxidase-*a*, but not for one containing little of this (Figs. 3, 4, 5, 7). The possibility that even the appearance of the peaks does not occur rapidly enough to be related to enzymic activity seems to be excluded by experiments with fast-reaction techniques (Bray, 1961).

Since signal *C* appeared on treatment of 'active' enzyme samples with xanthine, within 2 min. at 5°, its appearance may also be related to the catalytic activity (see also Bray, 1961).

The oxidation of DPNH is very slow, and is brought about by 'inactive' as well as by 'active' xanthine oxidase (Bergel & Bray, 1958). This is consistent with the present observation that DPNH caused only a slow development of signals *A* and *B*.

Nature of the changes occurring on reduction of xanthine oxidase-a

The peak in the xanthine titration curves (Figs. 3*a* and 7) suggests very strongly that both the flavin and the molybdenum of xanthine oxidase-*a* can be reduced by the substrate to two different levels. Thus reduction of flavin to the first level (FADH) gives signal *A*, and reduction to the second level causes this signal to disappear. Similarly, reduction of molybdenum to Mo(v) gives signal *B* and this also disappears on further reduction. This interpretation is supported by the fact that partial reoxidation of the enzyme with oxygen from a point to the right of the peak caused signals *A* and *B* to increase, whereas further reduction brought about by the addition of more xanthine caused peak-region signals to diminish.

The proportions of flavin and molybdenum in xanthine oxidase-*a* that are present in signal-giving forms during titration with the substrate are of interest. As stated above in one experiment the maxima corresponded to 0·07 and 0·23 equiv. of unpaired electrons/mole of total enzyme for signals *A* and *B* respectively, and 60% of the enzyme was in the *a* form. Hence for the xanthine oxidase-*a* (which contains 2FAD and 2Mo/mol.) about 6% of the FAD was present as the free radical and about 19% of the molybdenum as Mo(v), during the titration.

The conclusion reached in previous publications (Vänngård *et al.* 1961) that the formation of signals *A* and *B* did not involve the participation of oxygen is strengthened by the present data, which showed: first, that the signals were still obtained under improved anaerobiosis; secondly, that the effect of introducing a controlled amount of oxygen could be explained by assuming that this merely diminished the amount of xanthine available.

Since xanthine and purine gave peaks of about the same intensity (Figs. 3*a* and 4*a*) it follows that equal numbers of active centres are involved. Further, the positions of the maxima expressed in terms of reducing equivalents/mole of enzyme (1 mole of xanthine $\equiv 2e$ and 1 mole of purine $\equiv 6e$) are about the same for the two substrates. Therefore each active centre reacts with three times as many xanthine as purine molecules. It is unlikely that as many as three xanthine molecules would react at a single centre, and hence each purine molecule must react successively with more than one active centre. Hence the signals cannot be derived from enzyme–substrate complexes of the type discussed by Hollocher & Commoner (1960).

The facts that the flavin of the enzyme can be reduced beyond the signal-giving level and that it is never all present in this form can be explained by equilibria between the semiquinone free radical (FADH) and the oxidized (FAD) and fully reduced ($FADH_2$) forms:

$$FAD \overset{\pm 1e}{\rightleftharpoons} FADH \overset{\pm 1e}{\rightleftharpoons} FADH_2$$

For molybdenum the most obvious explanation would be analogous equilibria such as:

$$Mo(VI) \overset{\pm 1e}{\rightleftharpoons} Mo(V) \overset{\pm 1e}{\rightleftharpoons} Mo(IV)$$

However, reduction to the quadrivalent level is not the only possible cause for a Mo(v) signal's disappearing. For instance, the two Mo atoms of the enzyme could conceivably interact as in the diamagnetic dimer of Mo(v) reported by Sacconi & Cini (1954).

Since the peaks of the flavin and molybdenum curves (Figs. 3*a* and 7) are both at the same xanthine concentration, the redox potentials of the flavin and molybdenum systems of the enzyme must be very close. A more generalized equation for the reduction of the enzyme may therefore be written:

$$E \overset{\pm ne}{\rightleftharpoons} EH_n \overset{\pm me}{\rightleftharpoons} EH_{n+m}$$

in which E represents the 'resting' enzyme, EH_n the form containing the signal-giving species and n and m the number of reducing equivalents required, per mole of xanthine oxidase-*a*, to bring about the transformations. Theoretical curves for the proportion of the species EH_n at any point in such a reversible two-step reduction process may be calculated, if $m = n$, by using the equations of Michaelis & Schubert (1938), and this has been done in Fig. 7. Despite scatter of the experimental points, the calculations provide some further justification for assuming that a two-step reduction does take place.

Evidence about the chemical nature of the reduced forms of the enzyme might in principle be deduced from the values of n and m. In Fig. 7, the maximum occurred at $1{\cdot}4 \pm 0{\cdot}4$ moles of xanthine/mole of total enzyme and the proportion of xanthine oxidase-*a* was 50%; therefore $n = 6 \pm 2$; less-precise data from Fig. 3 (*a*) support this value. Although it would have to be reduced somewhat if substrate binding to 'inactive' forms of the enzyme took place, the value is probably still high enough to suggest that there may be two independent active centres in each molecule of xanthine oxidase; this would, of course, be consistent with the content of two Mo atoms and 2 FAD molecules/enzyme molecule. One possible interpretation of $6e$ reduction giving the highest signals would be by way of the following reaction in each molecule of xanthine oxidase-*a*:

$$2\,Mo(VI) \rightarrow 2Mo(V) \rightleftarrows Mo(VI) + Mo(IV)$$
$$2FAD \rightarrow 2FADH \rightleftarrows FAD + FADH_2$$
$$2Fe(III) \rightarrow 2Fe(II)$$

However, there are other possibilities and further speculation does not seem justified.

Our stoicheiometric results appear reasonably consistent with spectrophotometric titrations (Totter & Comar, 1956), especially if, as seems probable, the flavin figures of these workers are rather too high. However, since the spectra of the reduced forms of the enzyme described here have not so far been determined, detailed comparison is not possible.

Dithionite-induced signal changes

From the above, pure xanthine oxidase-*a* would be expected to behave in the same way in titration with dithionite as with xanthine, i.e. it should give signals only in the peak region (cf. Fig. 3*a*). It therefore seems that most of the signals obtained in the presence of dithionite must come from 'inactive' forms of the enzyme. It appears that reduction of the molybdenum of xanthine oxidase-i_1 to the quinquivalent level proceeds relatively slowly in the presence of either the substrate or dithionite; there is no evidence that substrates can reduce the molybdenum of 'inactive' enzyme beyond the signal-giving level, though excess of dithionite apparently did so very slowly. The slowness of this second stage explains the very intense molybdenum signals obtained under some conditions with low-activity samples. As stated above, we obtained the most intense signal *B* in a dithionite titration; the sample contained 60% of xanthine oxidase-i_1 and only 10% of xanthine oxidase-*a*, and the signal corresponded to about 1·9 equiv. of unpaired electrons/mole of total enzyme. Thus the signal was rather higher than would be required if all the molybdenum was in the quinquivalent state. Although the apparatus had been only roughly standardized, it is concluded that substantially all of the molybdenum of the xanthine oxidase-i_1 at least was in the signal-giving quinquivalent state.

The observation of striking differences between xanthine oxidase-*a* and -i_1 in their behaviour towards dithionite represents the first chemical, as opposed to enzymic, difference that has been observed. The two forms thus cannot differ merely in their ability to bind substrate molecules; pre-

sumably the bonding of the molybdenum must differ in the two species.

It is useful to re-examine the results of Bray *et al.* (1959). The surprising conclusion is that nearly all the signals in Fig. 1 of that paper must have come from 'inactive' rather than 'active' xanthine oxidase. The new results emphasize that extreme caution must be exercised in interpreting incomplete electron-spin-resonance data on an enzyme such as xanthine oxidase which, as normally prepared, consists of 'active' and 'inactive' forms.

The disappearance of signal *D*, which is due to impurities unrelated to any of the forms of xanthine oxidase, was brought about, not by xanthine, but by an excess of dithionite or by micro-organisms. Changes in this signal appear to be unrelated to the catalytic activity of xanthine oxidase.

Magnetic susceptibility; state of the iron

The resting enzyme gives no electron-spin-resonance signals from flavin or molybdenum, and its paramagnetism must therefore be due to the iron. Table 2 shows that the experimental value is compatible with 2–8 of the iron atoms' being tervalent, in the low-spin state. Some contribution from the high-spin state would be expected when the impurity responsible for signal *D* was present, since signals in the region of $g = 4$ would come from high-spin rather than low-spin forms of Fe(III). A single measurement on the sample showing a rather intense signal *D* and containing about 10% of *fa* indicated a more than doubled paramagnetic susceptibility of the resting enzyme, with diminished susceptibility increases on reduction. The resonance data give no evidence for low-spin Fe(III) in the resting enzyme. Bray *et al.* (1959) concluded that the iron of xanthine oxidase is in the ferrous state. However, the absorption by low-spin Fe(III) at $g = 2$ might easily be too broad to be detected by the present technique (cf. ferrihaemoglobin: Gibson, Ingram & Schonland, 1958; ferricyanide: Bleaney & Ingram, 1952).

The changes of susceptibility brought about by substrates or dithionite are generally too large to be accounted for by reduction to FADH and Mo(v) (Table 2), especially since, according to electron-spin-resonance data, only a portion of the flavin and molybdenum is ever in these paramagnetic forms. In the reduction by xanthine, which gave the smallest increase, the change could be explained by the formation of FADH, Mo(v) and Mo(III) in suitable proportions, if it is assumed that most of the metal is in the tervalent state. However, this is unlikely, and it is more probable that most of the increase in susceptibility with xanthine, purine or dithionite comes from reduction of low-spin ferric to high-spin ferrous atoms, this change being associated with the appearance of signal *C*. Thus at least part of the iron of the pure resting enzyme is in the ferric state and must be capable of reduction by xanthine to the ferrous state.

SUMMARY

1. Electron-spin-resonance and magnetic-susceptibility measurements have been carried out on xanthine oxidase alone and after the anaerobic addition of substrates or dithionite.

2. Four types of electron-spin-resonance signal have been observed. Two of these are the ones obtained previously and are attributed to the flavin–adenine dinucleotide semiquinone free radical (FADH) and Mo(v) respectively. The third signal has not been identified, but may be due to Fe(II) in the reduced enzyme; the fourth signal appears to be due to impurities.

3. An account is given of changes in the intensity of the FADH and Mo(v) signals that depend on the nature and concentration of the substrate and on the proportions of 'active' and 'inactive' xanthine oxidase. These take place only relatively slowly on adding the substrate. Differences between the 'active' and 'inactive' forms of xanthine oxidase are discussed.

4. It is concluded that 'active' xanthine oxidase can be reduced by the substrate to two different levels. The first of these shows the FADH and Mo(v) signals, but the second does not. The question of the nature of the reduced levels is discussed.

5. The strongest FADH and Mo(v) signals are, under some conditions, given by 'inactive' forms of the enzyme; it is concluded that in general incomplete electron-spin-resonance data on enzymes with variable specific activities should be interpreted with caution.

6. Susceptibility measurements indicate that, contrary to previous statements, some at least of the iron of resting xanthine oxidase is in the ferric state. Changes in susceptibility on reduction, together with the appearance of the unidentified signal, suggest that iron as well as flavin and molybdenum is reduced by the substrate.

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Sudden Freezing as a Technique for the Study of Rapid Reactions

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The kinetics of reactions in solution, having half-reaction times in the range of a few seconds down to 1 msec., are frequently studied by continuous-, stopped- or accelerated-flow methods. The 'quenching' method has been less widely used and differs from these methods in that the extent of reaction is measured at leisure on a series of samples taken from the streaming solution and 'fixed', by stopping the reaction after appropriate time intervals (Roughton & Chance, 1953). The essential requirement of this method is a quenching process which is rapid compared with the reaction being studied. Chemical quenching, in which the reaction is stopped by introducing a reagent that arrests the reaction at an appropriate point in the stream, has found limited application, but the principle of stopping a fast reaction by sudden cooling does not appear to have been considered. In this paper some of the conditions necesssary for the application of what may be termed the 'thermal quenching' method are discussed and a suitable apparatus is described. The usefulness of the method is limited by the availability of procedures for determining the extent to which the reaction has proceeded in the frozen samples. It was devised for use in conjunction with electron-spin-resonance measurements, which are in any case frequently made on frozen samples (see Addendum). The method has also proved suitable for the semiquantitative study of reactions involving colour changes.

Principle of the technique

Effect of cooling on reaction velocity. The technique depends on obtaining a series of samples of a mixture in which the reaction has been allowed to proceed for controlled periods and then stopped by suitable cooling. For a reaction dependent on molecular collisions and with a half-time of

100 msec., freezing within 10 msec. and holding the samples just below the freezing point until they had been examined would presumably be effective. However, intramolecular rearrangements might continue in the frozen state; e.g., with a constant temperature coefficient of 2 per 10° and a half-time of 100 msec. at 20°, the half-times would be about 2 min., 2 hr. and 2 days at temperatures of −80°, −140° and −190° respectively. It would therefore be necessary to cool the frozen samples from such reactions further, if possible to the temperature of liquid nitrogen, with little delay to obtain effective quenching.

Method of cooling. The most suitable method of cooling the solution appeared to be to squirt it through a narrow jet into an immiscible liquid maintained at as low a temperature as possible. This was preferred to squirting directly into liquid nitrogen (or into any liquid with a boiling point below the temperature of the reaction solution), since gasification of the coolant would retard heat transfer. The order of magnitude of time required to freeze, for example, a dilute aqueous solution at 20° squirted into hexane at −80° may be obtained by assuming that the water enters the hexane as a cylindrical stream with a diameter equal to the bore of the jet. If there is a large excess of hexane, and if sufficient turbulence is set up to maintain the surface of the water near to −80° throughout the freezing process, then the freezing time will depend solely on heat transfer through the water. Calculations based on these assumptions indicate that the freezing time goes up in proportion to the square of the bore of the jet and is about 10 msec. for a bore of 0·2 mm. The freezing time would be longer if air were carried into the hexane with the water, since this would delay the liquids' coming into contact with each other. The time would be shorter if the water broke up into smaller drops. It was concluded that solutions should be squirted through as narrow a jet as was practical, a theoretical upper limit for the bore being about 0·2 mm.

Method of driving the liquids. In order to conserve the solutions that are interacting, the flow of the liquids should be accelerated very rapidly to a predetermined value, maintained there until a sample large enough for measurement has been collected (this took a time of the order of 1 sec. in the final form of the apparatus) and then the flow stopped, again very rapidly; thus each sample would be essentially homogeneous with regard to the time that elapsed between mixing the solutions and freezing. This was achieved by driving the liquids from syringes by means of a constant-speed hydraulic ram. This type of apparatus is mentioned by Roughton & Chance (1953), but no details are given. The hydraulic pressure likely to be reached in the flowing liquid was calculated to be of the order of 10 atm. and the force required to drive the plungers of the syringes of the order of 3 kg. wt. If the hydraulic ram driving the plungers is not to be slowed down significantly by the syringes, it must be capable of delivering at least 10 times this force.

Details of the apparatus

The apparatus consisted of the following components: (i) the hydraulic ram unit; (ii) the syringes and reaction unit; (iii) the ram-velocity indicator. These were all mounted on a heavy steel base (Fig. 1).

Hydraulic ram unit. The ram *D* had a bore of 6 cm. and was mounted horizontally. It was connected via a magnetically operated valve *C* and a needle valve *B* to the bottom of a pressure vessel *A*. The vessel had a capacity of approx. 2 l. and was fitted at the top with a pressure gauge and two needle valves, one of which could be connected to a source of compressed air and the other to a vacuum pump. The pressure vessel was about one-third filled with aqueous glycerol (viscosity 8 centipoises) or, for some experiments, with methanol, which served as the hydraulic fluid. To operate the ram, the magnetic valve was closed and the lower needle valve *B* was opened to a suitable extent. Pressure (4 atm. for most experiments) was then applied to the vessel and the top valves were closed; on opening the magnetic valve, the ram moved forward at a virtually constant velocity. This velocity was controlled by varying the setting of the lower needle valve. The ram could be withdrawn by applying suction to the pressure vessel.

The operating end of the ram was fitted with two adjustable screw pieces *E* to bear on the syringe plunger heads and with a guide to prevent rotation of the ram. The travel of the ram was limited by means of a heavy adjustable screw-stop *F*. The stroke was terminated when the operating end of the ram hit this stop.

Syringes and reaction unit. This part of the apparatus is shown in Fig. 2. All-glass syringes were originally employed, but proved unsatisfactory for delivering concentrated protein solutions, since denaturation on the ground-glass surfaces caused the syringes to jam. They were therefore replaced by specially constructed syringes with Perspex barrels (3–6 mm. bore) and Teflon plungers. The connecting, reaction and jet tubes were 0·5 mm. bore polythene tubing with a wall thickness of 0·5 mm. The mixing chamber *H* was made by drilling three 0·5 mm. holes to meet at a point in a block of Perspex. (Slight imperfections in the drilling would improve the efficiency of mixing.) A number of interchangeable reaction tubes *K* with lengths varying from 10 to 90 cm. were used with the apparatus. Details of the connectors are shown in Fig. 3. Flanges of the shape illustrated could be made simply by heating the ends of the polythene tubes over a small electric radiant heater. Drilled-out stainless-steel screws going into tapped holes in the Perspex block served to press the polythene flanges firmly against the Perspex block. Thus no metal parts came into contact with the solutions. The jet *I* was made by drawing out a piece of polythene tubing over the electric heater. A certain amount of practice was required for this, and a satisfactory shape is shown in Fig. 4. Although the syringes were arranged horizontally, the jet actually pointed vertically downwards (i.e. into the plane of the paper in Fig. 2). For runs at the shortest possible

reaction time, the jet tube was connected directly to the mixing chamber.

Ram-velocity indicator. As the ram approached its stop *F*, a microswitch attached to it first closed and then opened a circuit which allowed a condenser to be charged up from a fixed-voltage source. The arrangement was such that the circuit closed when the ram was 9 mm. from the stop and opened again when there was 3 mm. of travel left. After the run, the voltage on the condenser was measured by switching it to a high-resistance internal-reflecting galvanometer. The condenser was normally charged to a potential low compared with the applied voltage; hence there was a linear relationship between the reciprocal of the galvanometer reading and the ram velocity. To calibrate the timer, the ram velocity was also measured directly, under suitable conditions, with a stop-watch.

In order to calculate the time interval corresponding to passage of a small portion of the solution from the mixing chamber to the jet, it was necessary to know the volumes of the reaction tubes and the jet tubes. These were conveniently determined by weighing each tube empty and full of water. The time t (msec.) could then be calculated from the equation $t = w/VA$, where V is the ram velocity (cm./sec.), A is the total cross-sectional area (cm.^{2}) of the two syringes and w is the weight (mg.) of water required to fill the reaction tube and jet. (The amounts of water in the mixing-chamber block and the block connecting the reaction tube to the jet were relatively small, and were ignored.)

Cooling methods. Two procedures were used. In the first, the reaction solution was squirted into hexane (m.p. −100°, b.p. +70°) or light petroleum (b.p. 60–80°) cooled

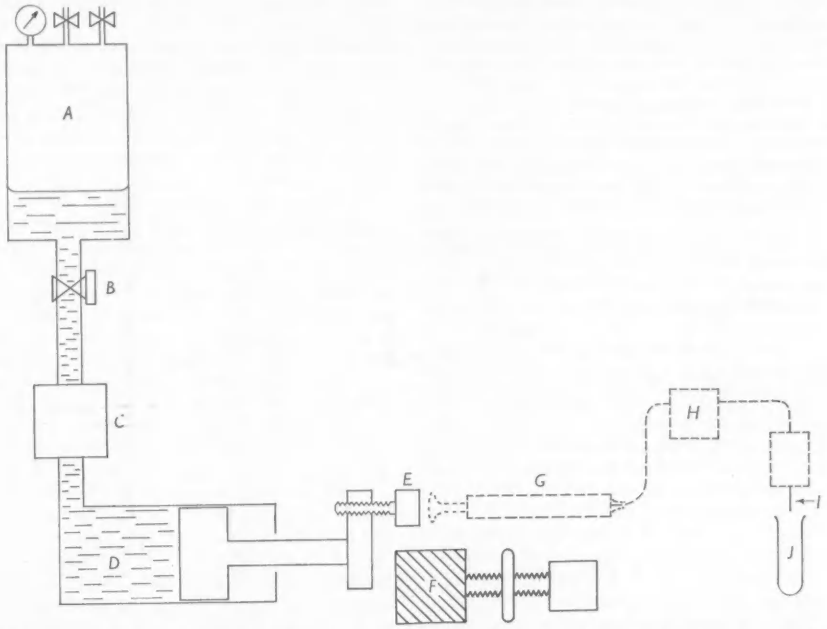

Fig. 1. Hydraulic system and general layout. *A*, Pressure vessel; *B*, needle valve; *C*, magnetic valve; *D*, hydraulic ram; *E*, adjustable screws bearing on syringe plunger heads; *F*, adjustable ram stop; *G*, syringes (the second is behind the first); *H*, mixing chamber; *I*, jet; *J*, container of cold liquid.

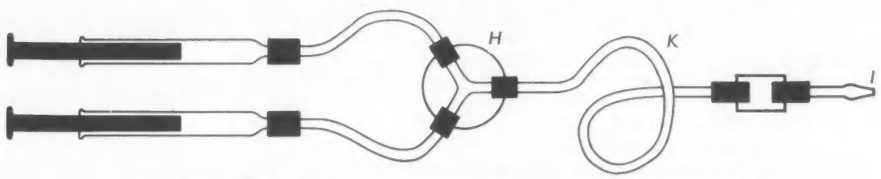

Fig. 2. Syringes and reaction unit. *H*, Mixing chamber; *I*, jet; *K*, reaction tube.

in solid CO_2 to about $-60°$. This proved satisfactory for some work, but on theoretical grounds a lower temperature seemed desirable. The second procedure thus consisted of squirting into *iso*pentane [m.p. $-160°$, b.p. $+30°$ (Harris, 1954)] at about $-140°$; below this temperature, the liquid became unduly viscous. The temperature of $-140°$ was maintained by means of a bath employing liquid nitrogen as the coolant.

Operating procedure. The syringes were filled, care being taken to avoid bubbles and any debris that might block the jet, and connected to the mixing chamber. The ram was brought slowly up to the syringes so that both screw stops *E* were in contact with the plungers, and the system was filled with the solutions up to the outlet from the reaction chamber. A suitable reaction tube and jet were then connected but left empty, and the ram stop *F* was set so that the required volume of solutions would be delivered. The ram was withdrawn a little way from the plungers (to ensure that it reached its maximum velocity before striking them) and the ram and timer controls were set. Immediately before the magnetic valve *C* was operated to make the run, a suitable container of the cooling liquid *J* was removed from its bath and held in the operator's hand, with the surface of the liquid 1–2 cm. below the jet. Squirting the aqueous solution into the hydrocarbon in this manner caused violent turbulence. The aqueous solution froze apparently instantaneously, to give a suspension of fine ice crystals which settled out within a few seconds. After the run, the container was returned to the cooling bath, and the timer was read.

In any series of experiments a fixed operating pressure and valve-setting were employed, so that the ram velocity was kept constant (or almost so) and the reaction time was varied by interchanging the reaction tubes *K*.

Tests on the apparatus

Flow tests. The ram velocity was fairly uniform and reproducible and relatively independent of the presence of the syringes. The smallest practicable jet had a bore of 0·10 mm.; a size of about 0·14 mm. was used for the quantitative work described below. Suitable flow rates were about 0·2–2·0 ml./sec., corresponding to flow velocities in the 0·5 mm. bore reaction tube of 1–10 m./sec. The lower limit was set by the performance of the ram and the jet and the upper limit by the strength of the polythene tubing. The range of reaction times covered by the apparatus was therefore about 10–1000 m-sec.

The efficiency of mixing chambers similar to the one described was established by Roughton & Chance (1953). The velocity for a change to turbulent flow in the reaction tube was about 4 m./sec. and most runs were carried out at lower velocities.

Tests on a model fast reaction. The reaction $H_2CO_3 \rightarrow H_2O + CO_2$ is one that has been used for testing other types of fast-reaction apparatus (Dalziel, 1953; Gibson, 1954). When 0·02 M-$NaHCO_3$ is mixed with 0·01 M-HCl, the solution is initially acid to bromophenol blue, but as the carbonic acid which is first formed decomposes,

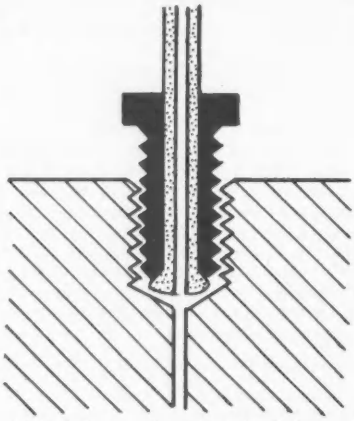

Fig. 3. Method of connecting a flanged polythene tube to a Perspex block.

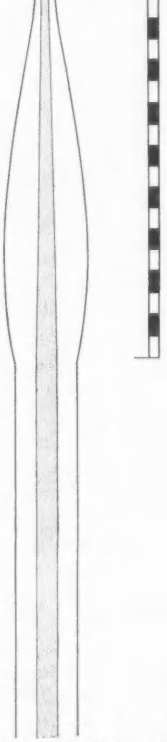

Fig. 4. Details of polythene jet *I* (see Fig. 1).

with a half-life of the order of 100 msec. at room temperature, the solution becomes alkaline to the indicator. Runs were carried out with the above concentrations of HCl and $NaHCO_3$ in the two syringes and with 0·02% bromophenol blue in each of the reagents (this indicator concentration is considerably higher than that used by the other workers).

Satisfactory results were obtained by using 150 ml. portions of light petroleum (b.p. 60–80°), contained in stoppered vessels and thoroughly cooled in solid CO_2–methanol. Preliminary experiments showed that with short reaction times the crystals were noticeably yellow, whereas with longer times they were bluish purple. On thawing, all the samples became purple. With many shapes of container, a small amount of solution often froze on the bottom rather than as free crystals. This material usually had a somewhat different colour from the bulk of the crystals, and had to be ignored when colour comparisons were made. It probably arose as a result of the petroleum's being stationary when the stream of water first struck it, allowing some of the water to pass straight to the bottom of the container rather than be carried around by the violent motion that ensued. The containers described in the Addendum did not seem to possess this disadvantage.

With the exception noted above the colour within a sample was always homogeneous. This is taken as evidence that in any one run the value of t was constant. Deviations would have been expected if the ram speed was not uniform, if there were air bubbles in the liquids, or if elasticity of the polythene tubing at the rather high operating pressures became a serious factor. When the jet bore was increased from 0·14 to 0·22 mm., which might have the effect of increasing the freezing time, noticeably bluer crystals were obtained.

Finally, an attempt to obtain semiquantitative results was made at 25° with reaction times in the range 20–300 msec. The frozen samples were kept in the bath for periods of 0·5–3 hr., then removed and photographed in colour. Once the samples were thoroughly frozen and cooled, no colour changes were observed. However, it was essential that the photographs were taken as soon as possible after the crystals were removed from the bath, since, apart from the danger of thawing, a reversible intensification of the colours appeared to take place on warming slightly. A series of citrate buffers of known pH containing bromophenol blue were prepared, and were run in the apparatus and photographed under identical conditions. It was then possible, by visual comparison of the photographs, to determine the pH at each value of t, generally with an estimated error of $\pm 0{\cdot}2$ pH unit. A graph of pH against t was plotted. Within the rather large limits of experimental error this graph was linear, cutting the $t = 0$ axis at pH $3{\cdot}8 \pm 0{\cdot}3$ and with a slope of 170 ± 40 msec./pH unit. These data are in satisfactory agreement with published work. Thus Dalziel (1953) found an initial pH of 3·5–3·6, whereas Gibson (1954) found 3·7. If 3·6 is assumed to be the correct value, then the time required to stop the reaction in the present work may be calculated to be 30 msec. (limits, 0–90 msec.).

It was therefore concluded that the performance of the apparatus was satisfactory and that at least one fast reaction could be effectively stopped by the cooling procedure described.

SUMMARY

1. Conditions necessary for stopping rapid reactions by squirting aqueous solutions through a fine jet into a cold immiscible liquid are discussed. An apparatus is described which produces homogeneous samples frozen at any desired stage of a reaction occurring in aqueous solution and having a half-life of the order of 100 msec.

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ADDENDUM

Electron-Spin-Resonance Measurements

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In order to make measurements of electron-spin-resonance signals on samples frozen by the technique described in the preceding (main) paper it was necessary to pack the ice crystals into tubes suitable for insertion into the cavity of a Varian V 4500 spectrometer. This was achieved by squirting a 0·3–0·5 ml. portion of the reaction mixture into about 40 ml. of cold hexane or *iso*pentane in a silica tube as illustrated in Fig. 1. The tube was returned to the cooling bath immediately after squirting and some of the ice crystals were packed into the bottom by working a packing device (Fig. 2) up and down in the tube, the latter being kept in the cooling bath throughout. A solid mass of crystals about 2 cm. in height was obtained at the bottom; the tube was then removed from the bath and the lower part immersed in liquid nitrogen until the electron-spin-resonance measurements were made. The operations, from squirting to cooling in liquid nitrogen, occupied 2–7 min. The measurements were made at the temperature of liquid nitrogen. The tubes were positioned carefully inside the vacuum flask of the spectrometer with the samples at the centre of the cavity. Comparison of the volumes occupied by the packed ice crystals and the water obtained on thawing indicated that about 60% of the packed volume was ice. This figure was fairly reproducible.

The technique was tested by using as a model reaction the reduction of Fe^{3+} to Fe^{2+} ions by quinol. This reaction has been studied by conventional methods by Baxendale, Hardy & Sutcliffe (1951), and extrapolation of their data suggested that a suitable reaction velocity would be obtained under the conditions set out in Fig. 3. The results in Fig. 3 show that reasonable precision was achieved.

The application of the method to the study of an enzymic reaction is reported by Bray (1961). It is interesting to compare the performance and potentialities, particularly for the study of enzyme intermediates, of the present freezing method with the more conventional continuous- and stopped-flow electron-spin-resonance methods. Continuous-flow methods suitable for examining free radicals arising from the substrate (Chance *et al.* 1961; Yamazaki, Mason & Piette, 1960) or from the enzyme (Commoner & Hollocher, 1960) have been described, but none of the procedures seem suitable for rapid-reaction work on enzyme intermediates. A stopped-flow method is also described by Yamazaki *et al.* (1960) and, by raising the enzyme concentration, this could in principle be extended to the observation of enzyme, in addition to substrate, free radicals. However, if the time resolution of the electron-spin-resonance spectrometer is reduced to the level necessary for such work, the

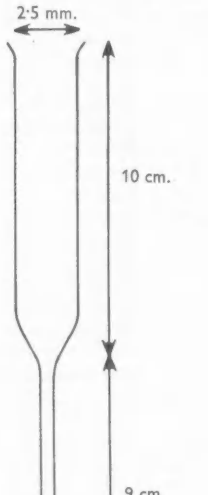

Fig. 1. Silica tubes. The diameters are internal.

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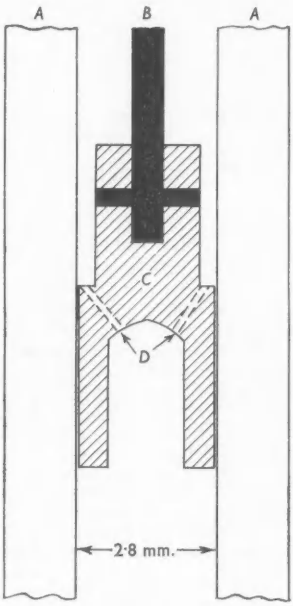

Fig. 2. Section through packing device. *A*, Lower part of silica tube (Fig. 1); *B*, stainless-steel rod extending to top of tube; *C*, Teflon packer, fitting fairly tightly in tube; *D*, small holes.

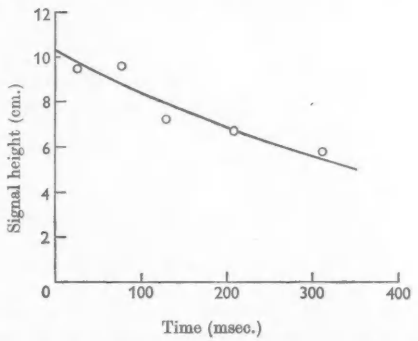

Fig. 3. Rate of decrease of electron-spin-resonance signal in the region of $g = 4$, on mixing a solution containing Fe^{3+} (0·04 g.atom/l. in 0·01 M-$HClO_4$ containing 0·5 M-$NaClO_4$) with an equal volume of quinol (0·4 M in the same solvent) at 25°. The reaction was stopped by squirting the solution into hexane at −60°. The curve through the experimental points was calculated for a unimolecular reaction with $k = 2{\cdot}0$ sec.$^{-1}$

signal:noise ratio falls; this means in principle that the faster the reaction, the higher the enzyme concentration required. In fact, in each of the four enzymic reactions studied by Bray (1961), Commoner & Hollocher (1960), Nakamura (1960) and Yamazaki *et al.* (1960), the lowest free-radical concentration reported was of the order of 0·1 μM. In general, though hyperfine structure may not be apparent in the cold, measurements on frozen aqueous samples give greater sensitivity than do those on unfrozen ones. Hence the present method should be capable of working at lower enzyme concentrations than the stopped-flow method. A further advantage of the freezing procedure is that certain signals which can readily be detected in the cold are not detectable at room temperature, presumably because of the rapid increase in line width (Beinert & Sands, 1960; Bray, Pettersson & Ehrenberg, 1961). The quantity of enzyme solution required to obtain an entire curve in the present procedure was about 2 ml., which is somewhat more than the minimum required for stopped-flow procedures, but very much less than that needed for continuous flow. Further, whereas the stopped-flow procedure has to be carried out successively at a series of fixed field strengths, each corresponding to a particular g, the freezing technique gives a complete electron-spin-resonance spectrum at each reaction time: this may be a considerable advantage where, for instance, new signals are unexpectedly encountered.

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The Chemistry of Xanthine Oxidase

8. ELECTRON-SPIN-RESONANCE MEASUREMENTS DURING THE ENZYMIC REACTION*

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Anaerobically, xanthine reduces the molybdenum, the flavin and probably also some of the iron of xanthine oxidase (Bray, Pettersson & Ehrenberg, 1961). In order to demonstrate that these changes are essential for the enzymic reaction, it is necessary to show that they occur at rates compatible with the overall kinetics of the enzymic reaction; the recent development of rapid-reaction techniques for electron-spin-resonance measurements has now made this feasible. This paper describes the application of the rapid-freezing technique (Bray, 1961) to the detection of enzyme intermediates during the enzymic oxidation of xanthine by molecular oxygen. Measurements, under conditions which would be expected to minimize interference by 'inactive' forms of the enzyme, have been made, both during the approach to and the decay from the 'steady state'; they provide strong evidence that the molybdenum, the flavin and possibly also the iron participate in the electron-transport processes of the enzyme and throw further light on the mechanism of the reaction.

EXPERIMENTAL

Xanthine-oxidase solutions. The samples, which were similar to those used by Bray *et al.* (1961), were dialysed against pyrophosphate buffer (pH 8·0–8·2) before use; calculations of the percentages of the different forms of the enzyme were made as described by Bray *et al.* (1961). Two samples, designated UXO-37 and UXO-43, were used for most of the experiments; they contained about 50 and 40% respectively of xanthine oxidase-*a*.

Rapid-reaction experiments. Resting xanthine-oxidase and oxygen-saturated solutions of xanthine were allowed to react together, from separate syringes, by the techniques described by Bray (1961) and Bray & Pettersson (1961). The reaction was quenched by squirting the reaction mixture into *iso*pentane at about 130°K. Electron-spin-resonance measurements were made at 77°K. No evidence of changes in the signals during storage of the samples in liquid nitrogen was observed; the time intervals from squirting to measuring ranged from about ½ to 20 hr. The spectrometer was standardized with a solution of copper–ethylenediaminetetra-acetic acid complex and the signals were measured as described by Bray *et al.* (1961). Corrections for variations in the tube diameters and also, where necessary, for incomplete coverage of the cavity by the packed sample, were applied.

The procedure was apparently without any harmful effects on the enzyme. On thawing out the frozen samples under *iso*pentane, no signs of precipitation could be detected; the ratio activity/E_{450} after thawing was at least 90% of that of the starting solution in measurements on UXO-37.

RESULTS

Electron-spin-resonance signals of varying intensities were obtained under all the conditions tested. Fig. 1 shows a typical trace, in which the signals, previously designated as *A*, *B* and *C* and attributed respectively to the flavine–adenine dinucleotide free radical (FADH), Mo(v) and perhaps Fe (II) (Bray *et al.* 1961), are clearly visible. In addition, weak signals (marked as *C′* in Fig. 1) are visible on the low-field side of signal *A*; like signal *C*, these resembled components of the unknown signal of Beinert & Sands (1960; see particularly their Fig. 3*e*), and presumably the two signals are closely related to one another. Signals *C* and *C′* could be distinguished clearly in almost all the rapid-reaction experiments that have been carried out, including some at short reaction times, but quantitative measurements of their intensities were not attempted.

Figs. 2–4 show the variations in the intensity of the Mo(v) signal as a function of the reaction time for the two preparations of xanthine oxidase. In Figs. 2 and 3 xanthine was present at a concentration comparable with that of the enzyme and the oxygen was present in excess. In Fig. 4 the substrate was present in excess over both enzyme and oxygen.

The striking difference between the two preparations (Figs. 2 and 3) was unexpected, since the concentrations of the enzyme, substrate and electron

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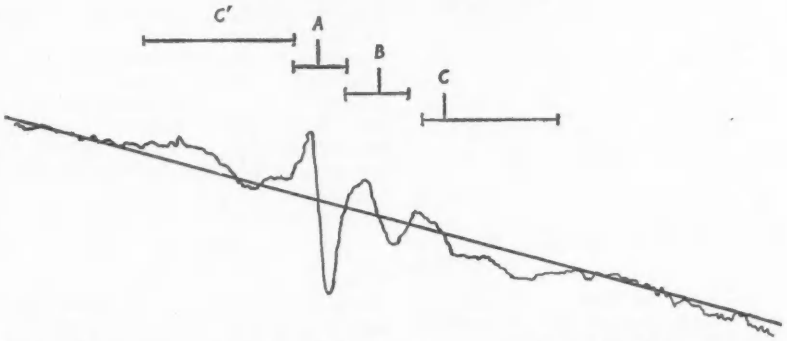

Fig. 1. Electron-spin-resonance curve (first derivative) for a sample of xanthine oxidase (UXO-43) frozen 100 msec. after mixing with oxygenated xanthine. For meaning of *A*, *B*, *C*, *C'* see text.

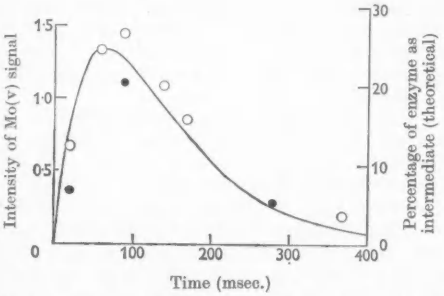

Fig. 2. The intensity of the Mo(v) signal (signal *B*) for samples of xanthine oxidase (UXO-43) frozen at various times after mixing with oxygenated xanthine at 24°. The signal-intensity units are arbitrary but are approximately the same and refer to the same concentration of total xanthine oxidase in Figs. 2–4. The concentrations immediately after mixing were: total xanthine oxidase, 0·08 mM; xanthine, 0·06 mM; oxygen, approx. 0·6 mM. The open (○) and full (●) circles refer to two different series of experiments; the theoretical curve was calculated as described in the Discussion from the velocity constants of Gutfreund & Sturtevant (1959).

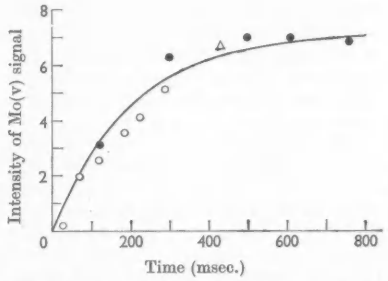

Fig. 3. As for Fig. 2, but with UXO-37 (concentration of total xanthine oxidase, 0·11 mM), at 30°. The theoretical curve was calculated for a unimolecular reaction with $k = 5$ sec.$^{-1}$ △; ○ and ● refer to different series of experiments.

acceptor were similar, as also were the compositions of the enzyme samples. Further, the histories of the two samples were almost identical; possibly the most significant difference was that the dialysis of UXO-37 before use was less exhaustive than that given to UXO-43. Since it was possible that the difference between the preparations might be due to an inhibitor in UXO-37, the effect of substrate concentration on the enzymic activities was investigated, with xanthine as substrate and oxygen as electron acceptor (Bray, 1959). In the presence of about 0·03 μM-xanthine oxidase, reaction rates were maximal at about 0·05 and 0·15 mM-xanthine respectively for UXO-43 and UXO-37. This difference is strong evidence for the presence of an inhibitor in UXO-37 which is absent from UXO-43 (Bray, 1959).

In addition to the Mo(v) signals, free-radical signals were also observed in all the rapid-reaction runs. In general, the form of the free radical/time curves followed the corresponding molybdenum curves fairly closely. The approximate average free radical/Mo(v) ratios for the points in Figs. 2–4 were respectively 1·0, 0·1 and 0·1 (these ratios have been corrected to correspond to equal areas under the absorption curves and therefore approximate to molecular ratios). The maximum observed concentrations of Mo(v) calculated for the experiments in Figs. 2–4 corresponded respectively to about 0·02, 0·1 and 0·1 g.atom/mole of total xanthine oxidase. Since the Mo(v) signals, particularly for UXO-43, were thus relatively weak, it was thought advisable to check that added inorganic molybdenum

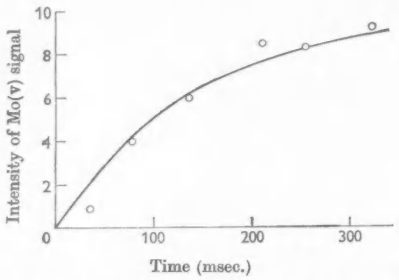

Fig. 4. As for Fig. 3, but with the xanthine concentration increased to 1·9 mM. The theoretical curve was calculated for a unimolecular reaction with $k = 7$ sec.$^{-1}$

did not give signals under the conditions of the experiments. Accordingly a sample of the enzyme was incubated with 1 mol.prop. of sodium molybdate for two days at 5° before running fast-reaction experiments side by side with runs on the untreated enzyme. The fairly weak signals obtained appeared identical in the control and treated samples.

Further evidence that the observed electron-spin-resonance signals are related to the enzymic activity and not to artifacts is provided by the observation that, after thawing samples for a few minutes and then refreezing, signals could no longer be detected. This observation was made with UXO-37, by using an excess of oxygen over xanthine.

DISCUSSION

Gutfreund & Sturtevant (1959) used rapid spectrophotometric techniques to follow changes in the absorption at 450 mμ during the xanthine oxidase-catalysed oxidation of xanthine by molecular oxygen. They concluded that at 25° a rapid combination of enzyme and substrate was followed by a unimolecular reduction with $k_{red.}$ 10·5 sec.$^{-1}$, the reduced enzyme then combining rapidly with oxygen and being reoxidized with a unimolecular constant, $k_{ox.}$, of 21·5 sec.$^{-1}$

If it is assumed that there are two active centres/mole of xanthine oxidase-*a* (Bray *et al.* 1961), then in Figs. 2 and 3 the concentration of enzyme active centres was the same as, or slightly greater than, the substrate concentration; further, the electron acceptor was present in excess. No true steady state would be expected under these conditions; if the Gutfreund & Sturtevant mechanism is accepted, the reaction would be represented simply by two successive unimolecular reactions:

$$\mathrm{ES} \xrightarrow{k_{red.}} \mathrm{EH} \xrightarrow{k_{ox.}} \mathrm{E},$$

where ES is the enzyme–substrate complex, EH is a reduced form of the enzyme and E is the resting, reoxidized enzyme. For such a system, the concentration of the reduced form, [EH], can readily be plotted as a function of time if the velocity constants and the initial concentration of the enzyme–substrate complex $[ES]_0$ are known (Frost & Pearson, 1956). With Gutfreund & Sturtevant's velocity constants, the maximum concentration of EH should occur at 65 msec. with

$$[EH]/[ES]_0 = 25\,\%$$

at this point. The theoretical curve calculated in this manner is shown in Fig. 2 (solid line), with an arbitrary concentration scale. Considering the relatively low precision of the measurements on the rather weak Mo(v) signals from UXO-43 and the fact that the velocity constants were obtained by a totally different technique in another Laboratory, the agreement between the experimental points and the theoretical curve is surprisingly good; it provides strong evidence that the Mo(v) signals in this instance are associated with the reduced form of the enzyme.

As stated above the experimental maximum in Fig. 2 corresponded to about 0·02 g.atom of Mo(v)/mole of total xanthine oxidase. It was reported by Bray *et al.* (1961) that the peak signals associated with the reduced form of xanthine oxidase-*a*, designated 'EH_n', corresponded in one experiment to 0·23 g.atom of Mo(v)/mole of total enzyme. Thus if it is assumed that the molybdenum signals arise, as previous work would lead one to expect, from this 'first reduced level' of the enzyme, 'EH_n', then the maximum proportion of the active enzyme in this form in Fig. 2, after correction for the differing contents of xanthine oxidase-*a* in the two experiments, would be $100 \times 0{\cdot}02/0{\cdot}23 \times 60/40$, i.e. 13 %. As shown above, the theoretical figure from Gutfreund & Sturtevant's constants is 25 %. Since the calculations are approximate, the agreement between the experimental and theoretical values may be regarded as satisfactory.

Since for UXO-43 the data on the Mo(v) signal are entirely consistent with the metal's participating in the electron transport, it is all the more surprising that, for UXO-37, a very similar sample which was examined under similar conditions, the signal, instead of showing a maximum in the region of 65 msec., continued to rise up to about 400 msec. and then remained constant for at least a further 400 msec. (Fig. 3). The plateau corresponded to about 0·1 g.atom of Mo(v)/mole of total xanthine oxidase, which is very roughly what would be expected if all of the xanthine oxidase-*a* was in the 'first reduced level'. Thus it seems that here, for some reason, reoxidation of the enzyme is not taking place, or rather, since the signals disappeared

on thawing and refreezing, that reoxidation is taking place only slowly. Gutfreund & Sturtevant (1959) also observed that reoxidation of the reduced enzyme at the end of a steady-state period was slow, but they interpreted this as being due to hydrogen peroxide formed in the reaction. A negligibly low rate of reoxidation in the present fast-reaction experiments is not necessarily inconsistent with the observed high activity in the ordinary assay procedure, since the fast-reaction work was done at an abnormally high enzyme concentration. At such concentrations, the presence of endogenous inhibitors, which could be almost without effect in the assay of the xanthine oxidase-*a* content of UXO-37, might stop the reaction almost completely (Bray, 1959).

The proposition that for UXO-37 the plateau in Fig. 3 represents complete reduction of xanthine oxidase-*a* to the first reduced level is strengthened by the fact that almost exactly the same curve was obtained when xanthine was in excess over oxygen (Fig. 4). Further, the rate of appearance of the Mo(v) signal was not greatly affected by variations in substrate concentration (Figs. 3, 4). This is consistent with the rate-limiting step's being reduction of the enzyme–substrate complex, as the theory requires.

It was stated by Bray *et al.* (1961) that xanthine oxidase-*a* can be reduced to a 'second reduced level' designated as 'EH_{n+m}', which did not give electron-spin-resonance signals. However, since all the results in the present paper can be explained satisfactorily in terms of reduction to the first level only, no further evidence on this can be deduced. Since, in the presence of excess of xanthine (Fig. 4), the signals showed no signs of decreasing, up to 300 msec. at 30°, reduction to this second level must be a relatively slow process, although previous work showed it to be complete within 2 min. at 4° (Bray *et al.* 1961). Possibly sluggishness in the reactions of the second reduced level could account for the well-known phenomenon of inhibition of xanthine-oxidase activity by excess of substrate.

No explanation has been given here for the variations in the free radical/Mo(v) ratio, and the nature of the inhibitor postulated to explain the batch variations is still unknown. Further, the weakness of the Mo(v) signals during the reaction was explained by equilibria within the 'first reduced level' (Bray *et al.* 1961), but the nature of the equilibria is still not understood. Nevertheless, the appearance and disappearance of molybdenum signals has been correlated with published kinetic data and the signals were obtained with an excess of enzyme over substrate, by using high concentrations of the electron acceptor, i.e. under conditions that should preclude interference by 'inactive' forms of the enzyme. Incubation of the enzyme with inorganic molybdate before the experiments had no influence on the results. It is concluded that the results represent the first direct evidence for a redox function for the molybdenum of xanthine oxidase. Since signals believed to arise from iron reduction were also observed, this metal may also participate in the electron transport. No evidence on the possible sequence of the reduction of the metals and flavin of xanthine oxidase is at present available.

SUMMARY

1. Electron-spin-resonance signals obtained from xanthine oxidase during the enzymic oxidation of low concentrations of xanthine by molecular oxygen have been studied with a fast-reaction technique.

2. Two preparations of xanthine oxidase were examined. For one, the kinetics of the appearance and disappearance of the Mo(v) signal correlated well with the known kinetics of the overall reaction, thus providing the first direct evidence for the participation of the metal in the electron-transport processes of the enzyme. Reoxidation of the second sample at the high enzyme concentrations needed for electron-spin-resonance measurements was slow; this may have been due to the presence of an inhibitor.

3. The detection of signals possibly arising from the reduction of iron may indicate a redox function for this metal in addition to flavin and molybdenum in xanthine-oxidase action.

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Respiratory Systems in the Trypanosomidae

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At least two different types of hydrogen-transport system must be present in flagellates of the family Trypanosomidae (for a classification, see Hoare, 1957) since some species do not contain detectable cytochrome pigments and their respiration is insensitive to cyanide, azide (Brand, 1951; Ryley, 1956) and carbon monoxide (Fulton & Spooner, 1959). These differences may be observed not only among different species which infect the blood of vertebrates, but also between different forms of the same strain, whose life cycle involves both invertebrate (insect) and vertebrate stages. The present work is concerned with the nature and distribution of respiratory enzymes and enzyme systems present in subcellular fractions obtained from some of these protozoa.

MATERIALS AND METHODS

Blood-stream forms. The 'L' strain of *Trypanosoma rhodesiense* (Grant & Fulton, 1957) was isolated from a patient in 1923 and has subsequently been maintained in rodents by blood inoculation. *Trypanosoma vivax* was the strain described by Desowitz (1956), which had been adapted to the rat as experimental host. *Trypanosoma lewisi* was isolated in 1956 from a natural infection of a wild rat. *Trypanosoma congolense* (Busimbi strain) was isolated from cattle in Buganda, East Africa, in 1945, and subsequently maintained in rodents. The following strains were received from the Wellcome Laboratories of Tropical Medicine through the kindness of Dr R. A. Neal: *Trypanosoma gambiense* (Wellcome C.L.) isolated from a patient in Liberia in 1939 and subsequently maintained in rodents; *Trypanosoma evansi* (Wellcome N.S.) isolated from a camel in the Sudan in 1938 and subsequently maintained in mice; *Trypanosoma equinum* (Wellcome Z.U.) obtained from the Argentine Public Health Authorities in 1948 and subsequently maintained in mice.

Groups of infected rats were killed at the height of infection and trypanosomes isolated from the blood by the procedures detailed by Fulton & Spooner (1959). Microscopic examination of each suspension was made on smears treated with Giemsa stain, and the suspension was only retained for experimental use if the content of platelets and white and red blood cells was negligible.

Culture forms. Details of the strain of *Strigomonas oncopelti* and its cultivation were given by Ryley (1955). Cultures of *T. rhodesiense* (Wellcome C.T.) and *T. gambiense* (Cheick) were obtained from Dr T. von Brand; they were cultivated in the medium described by Tobie, Brand & Mehlman (1950) except that citrated bovine blood was used for large-scale culture. Usually 36 or 72 conical flasks of 250 ml. capacity, each containing 25 ml. of blood–agar base and 15 ml. overlay, provided sufficient material for any one experiment; cultures were harvested after 6 days' growth at 24°. *Trypanosoma cruzi* and *Leishmania donovani* were culture strains of unknown origin; they were cultivated for 14 days at 24° in the diphasic medium described by Johnson (1947), by using citrated bovine blood. In all cases, the flagellates were isolated from the liquid phase of the medium by centrifuging at 1000*g* for 10 min., and the sedimented cells were washed twice in 0·9% NaCl.

Cell-counting procedure. In some experiments, a portion of the suspension of trypanosomes was diluted with a solution containing formaldehyde and crystal violet (Voegtlin & Smith, 1920), and the stained flagellates were counted in a haemocytometer chamber with improved Neubauer ruling.

Preparation of cell-free extracts. Blood-stream forms were lysed by suspension in water as described by Grant & Sargent (1960). The suspension was then centrifuged at 1000*g* for 10 min. and the clear supernatant was removed. The residue, which contained about 90% of the total N present in the starting material, was resuspended in water. This suspension was centrifuged, the supernatant discarded, and the washed residue resuspended in water as before. All these procedures were carried out at 2°. The final suspension was used experimentally within 30 min. of its preparation.

Culture forms became swollen but did not lyse when suspended in distilled water (4 vol.). Cell-free preparations were, however, obtained by cooling these suspensions to −70° with subsequent thawing in a water bath at 30°. This freezing and thawing procedure was repeated, and the suspension of completely ruptured cells was then diluted with distilled water (6 vol.) and centrifuged at 1000*g* for 10 min. The supernatant was discarded and the residue resuspended in distilled water. This final suspension (2–5 mg. of N/ml.) was used experimentally within 30 min. of the initial cooling of the intact cell suspension to −70°.

In some experiments (Table 6), enzyme preparations were obtained by freeze-drying suspensions of intact cells of either blood-stream or culture forms. The freeze-dried material was subsequently suspended in distilled water (1–6 mg. dry wt./ml.) with the aid of an all-glass homogenizer. The particulate material obtained after centrifuging

at 1000*g* for 10 min. was again resuspended in water. After centrifuging, the supernatant was discarded and the residue resuspended in water as before.

Oxygen uptake. Consumption of O_2 was measured in conventional Warburg apparatus in air at either 30° or 37°. The fluid volume added to each flask was 3 ml. and this included 0·1 ml. of 40% (w/v) KOH contained in the centre well. In experiments where cyanide was used as an inhibitor, KOH was replaced by the KOH–KCN or $Ca(CN)_2$–$Ca(OH)_2$ mixtures recommended by Robbie (1946).

Total nitrogen. Total N was determined by the micro-Kjeldahl method; the NH_3 produced was distilled into a boric acid solution containing methyl red–bromocresol green indicator or alternatively estimated directly with Nessler reagent (Conway, 1950).

Materials. DL- and L-α-Glycerophosphate were synthesized and isolated as the sodium salts (Grant & Sargent, 1960). DPNH, mammalian cytochrome *c* and tris were obtained from C. F. Boehringer und Soehne GmbH., Mannheim, Germany. Phenazine methosulphate obtained from the Aldrich Chemical Corp. Inc., Milwaukee, Wis., U.S.A., was recrystallized twice from ethanol before use. Dihydroxyacetone phosphate was prepared from a synthetic sample of the *cyclo*hexylamine salt of the dimethyl ketal of dihydroxyacetone phosphate (Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.) as described by Ballou & Fischer (1956). Antimycin A was a gift from Dr E. F. Hartree.

RESULTS

Respiratory systems in blood-stream forms

In previous experiments with *T. rhodesiense* (Grant & Sargent, 1960), the most active cell-free preparations were obtained by lysis of the cells with distilled water. In these suspensions the particulate material contained the substrate-specific L-α-glycerophosphate-oxidase system and this mode of preparation was accordingly used with other strains of the *brucei* group (Table 1). The term *brucei* is used to denote a group of organisms which on morphological and phylogenetic evidence are closely related to *Trypanosoma brucei*. Under optimum conditions of pH and substrate concentration, the initial rate of oxygen uptake by these particulate preparations in the presence of either succinate or DPNH was less than 5% of that found with L-α-glycerophosphate; the addition of catalase (0·5 μM) did not decrease the rate of oxygen uptake. It was concluded that all strains of the *brucei* group contained a hydrogen transport system of similar activity which had a marked specificity for L-α-glycerophosphate as substrate.

The supernatants obtained by centrifuging lysed cell suspensions had no oxidase activity towards either L-α-glycerophosphate or DPNH. However, the addition of both the particulate fraction and DPNH to the corresponding supernatant resulted in an appreciable uptake of oxygen (Table 2). This effect was abolished by prior dialysis of the supernatant, but the uptake of oxygen was largely restored by the addition of trace amounts of either dihydroxyacetone phosphate or L-α-glycerophosphate. Dihydroxyacetone phosphate and a DPN-linked L-α-glycerophosphate dehydrogenase have both been shown to be present in the cytoplasm of *T. rhodesiense* (Grant & Sargent, 1960). It is therefore probable that the oxidation of DPNH by the combined cytoplasmic and particulate fractions of these cells can be attributed to the coupled functioning of the reactions catalysed by the particulate L-α-glycerophosphate oxidase and the cytoplasmic DPN-linked dehydrogenase. The oxidation of DPNH would thus involve indirect hydrogen transfer to the respiratory chain via L-α-glycerophosphate.

The oxygen uptake of particulate preparations obtained in a similar manner from blood-stream

Table 1. *Effect of substrates on the oxygen uptake of cell-free preparations of blood-stream forms*

Reaction mixtures contained 'water-lysed' preparation (approx. 0·5 mg. of N), tris buffer, pH 7·6 (0·14 M), and substrate in a final volume of 3·0 ml. The concentrations of substrates, added either singly or together, were L-α-glycerophosphate (5·5 mM), succinate (6·2 mM), cytochrome *c* (1·0 mg./flask) and DPNH (7·6 mM). Uptake of O_2 was measured at 37° over a 45 min. period except with L-α-glycerophosphate when readings were discontinued after 25 min. Negligible O_2 uptake was observed in the absence of substrate. – Signifies value not determined.

		Uptake of O_2				
		(μl./hr./10^8 cells)	(μl./10 min./mg. of N)			
Group	Species	L-α-Glycerophosphate	L-α-Glycerophosphate	Succinate	Succinate + cytochrome *c*	DPNH
brucei	*T. rhodesiense*	89	118	<5	<5	<5
	T. gambiense	69	111			
	T. evansi	63	99			
	T. equinum	–	101			
vivax	*T. vivax*	–	35	10	12	8
congolense	*T. congolense*	–	19	9	9	22
lewisi	*T. lewisi*	–	20	17	28	19

forms of other groups (Table 1) showed that there was an appreciable direct oxidation of DPNH and succinate as well as of DL-α-glycerophosphate.

Effect of inhibitors. The strain of *T. lewisi* was the only blood-stream form used in these experiments which contained detectable cytochrome pigments (Ryley, 1951; Fulton & Spooner, 1959); cell-free preparations contained succinoxidase activity which was enhanced by the addition of cytochrome *c* (Table 1) and was inhibited by both cyanide and antimycin A. The degree of inhibition was largely independent of the substrate used (Table 3). In contrast, the oxidation of L-α-glycerophosphate by particulate preparations obtained from all strains of the *brucei* group was unaffected by cyanide and antimycin A at the concentrations of these reagents given in Table 3.

Respiratory systems in culture forms

Unlike blood-stream forms, cells cultivated *in vitro* did not lyse when suspended in water. Cell-free preparations were, however, obtained by freezing and thawing these latter suspensions. Oxidase activity was confined to the particulate material and the initial rates of oxygen uptake at 30° in the presence of various substrates are given in Table 4. DL-α-Glycerophosphate and succinate were both oxidized, but the highest rate was observed in the presence of DPNH, the ratio of these rates being about 1:2:5 respectively.

Included in Table 4 as a direct comparison with these results are corresponding data obtained with particulate preparations of blood-stream forms obtained by the same freezing and thawing procedure. At 30°, the rate of oxygen uptake in the presence of DL-α-glycerophosphate was appreciably greater than that found with either succinate or DPNH. This result is in general agreement with those given in Table 1, although the freezing and thawing procedure clearly had an adverse effect on the activity of these enzyme systems, the effect on L-α-glycerophosphate oxidase being particularly marked. Under the conditions given in Table 1, oxidase activity at 30° was only about 5–10% lower than that measured at 37°. For these reasons it seemed probable that oxidase activity in preparations of the culture forms was also partly inactivated by the freezing and thawing procedure. This possibility has been further studied by the measurement of α-glycerophosphate and succinic-dehydrogenase activity in freeze-dried preparations of these cells (Table 6).

Particulate preparations of the culture form of *T. cruzi* oxidized DPNH at an appreciable rate; this rate was not influenced by the addition of the cytoplasmic fraction of the cell homogenate as was found with the blood-stream form of *T. rhodesiense* (Table 2).

Effect of inhibitors. With particulate preparations obtained from *T. rhodesiense*, *T. gambiense* and *S. oncopelti*, both cyanide and antimycin A had a marked inhibitory effect on the rate of oxygen uptake when DL-α-glycerophosphate or succinate were added as substrates. In contrast, the rate of oxidation of DPNH was not inhibited by cyanide or by antimycin A (Table 5); rather stimulation of the rate of oxygen uptake was observed and this effect was particularly marked with cyanide.

Table 2. *Oxidation of reduced diphosphopyridine nucleotide by cell-free fractions from blood-stream forms of* Trypanosoma rhodesiense

Intact cells (6 mg. of N) were lysed in distilled water (20 ml.) at 2°. The suspension was centrifuged and a portion of the supernatant was dialysed against distilled water for 4 hr. at 2°. The particulate material was washed and re-suspended in distilled water (18 ml.). Reaction mixtures contained tris buffer, pH 7·6 (0·14 M), particulate suspension (1 ml.) and the additions as shown below in a total volume of 3 ml.

Addition	Uptake of O_2 (μl./flask/25 min.)
DPNH (22 μmoles)	<5
Supernatant (1 ml.)	<5
Supernatant (1 ml.) and DPNH (22 μmoles)	58
Dialysed supernatant (1 ml.) and DPNH (22 μmoles)	<5
Dialysed supernatant (1 ml.), DPNH (22 μmoles) and dihydroxyacetone phosphate (2 μmoles)	47
Dialysed supernatant (1 ml.), DPNH (22 μmoles) and L-α-glycerophosphate (2 μmoles)	50

Table 3. *Effect of inhibitors on the oxygen uptake of cell-free preparations of* Trypanosoma lewisi

Experimental details were as described in Table 1. Enzyme (0·9 mg. of N) was equilibrated for 10 min. with either antimycin A (5 μg./flask) or cyanide (0·5 mM) before the reaction was started by tipping substrate from the side arm. Antimycin A was added as a solution (0·1 ml.) in aqueous 50% (v/v) ethanol.

Substrate	Uptake of O_2 (μl./30 min./flask)		
	Control	Cyanide	Antimycin A
L-α-Glycerophosphate	53	24	43
Succinate and cytochrome *c*	71	27	48
DPNH	49	16	35

Dehydrogenase activity of particulate preparations

Although a cytoplasmic L-α-glycerophosphate dehydrogenase was present in blood-stream forms of the *brucei* group, this enzyme was DPN-linked and was distinct from the particulate L-α-glycerophosphate dehydrogenase whose activity could be determined with phenazine methosulphate as electron acceptor. Optimum conditions for these assay procedures were determined as described by Grant & Sargent (1961).

Suspensions of the particulate material obtained in an identical manner from freeze-dried samples of either blood-stream or culture forms could be repeatedly frozen and thawed or stored for several days at 2° without significant loss in L-α-glycerophosphate- or succinic-dehydrogenase activity. In the assay of these enzymes with phenazine methosulphate as electron acceptor, 1 mole of oxygen was taken up/mole of substrate utilized (Kearney & Singer, 1956; Grant & Sargent, 1961), so that the rates of oxygen uptake given in Table 6 should be halved to make them comparable with those of the corresponding oxidase systems (Tables 1 and 4). In this way it can be seen that the ratio of dehydrogenase to oxidase activity in blood-stream forms (Table 1) was about 2:1, whereas the ratio was about 3:1 for the culture forms of *T. rhodesiense* and *S. oncopelti* and about 5:1 for the culture form of *T. gambiense* (Table 4). These results suggest that about half of the succinic- and L-α-glycerophosphate-oxidase activity of blood-stream forms had been inactivated during the isolation procedure and that this effect was even greater during the freezing and thawing procedure used to obtain cell-free preparations of the culture forms. Nevertheless, the ratio of activities of succinic to α-glycerophosphate dehydrogenase in preparations of any one strain (Table 6) was similar to that of oxidase activities in preparations of the same blood-stream (Table 1) or culture strain (Table 4). This indicated that inactivation of oxidase activity during the isolation procedure was a general rather than a selective effect on one particular oxidase system.

Table 4. *Effect of substrates on the oxygen uptake of 'frozen and thawed' cell-free preparations*

Reaction mixture contained 'frozen and thawed' preparations (2–5 mg. of N), tris buffer, pH 7·3 (0·14 M), and substrate in a final volume of 3 ml. The concentrations of substrates added either singly or together were DL-α-glycerophosphate (10·0 mM), succinate (5·3 mM), cytochrome *c* (1 mg./flask) or DPNH (5·3 mM). Initial rate of O_2 uptake was measured at 30° for 30 min. Negligible O_2 uptake was observed in the absence of substrate. (*B*) and (*C*) indicate blood-stream and culture forms respectively.

Species	Uptake of O_2 (μl./10 min./mg. of N)			
	DL-α-Glycero-phosphate	Succinate	Succinate + cytochrome *c*	DPNH
T. rhodesiense (*C*)	3·4	6·1	6·6	17·4
T. gambiense (*C*)	2·3	3·4	4·0	14·4
T. cruzi (*C*)	1·2	2·8	3·6	7·2
L. donovani (*C*)	<1·0	4·5	6·0	7·2
S. oncopelti (*C*)	1·9	3·2	3·4	12·2
T. rhodesiense (*B*)	17·9	1·2	1·6	3·5
T. evansi (*B*)	16·8	1·4	1·6	3·2
T. equinum (*B*)	15·3	<1·0	<1·0	2·1
T. vivax (*B*)	5·5	<1·0	1·6	2·9
T. congolense (*B*)	3·4	<1·0	<1·0	2·5

Table 5. *Effect of inhibitors on the oxygen uptake of cell-free preparations of culture forms*

Reaction mixtures contained 'frozen and thawed' preparations (2·5, 2·2 and 6·9 mg. of N/flask for *T. rhodesiense*, *T. gambiense* and *S. oncopelti* respectively) and the other components detailed in Table 4. Enzyme was equilibrated for 10 min. with either antimycin A (5 μg./flask) or cyanide (0·5 mM) before the reaction was started by tipping substrate from the side arm.

Substrate	Inhibitor added	Uptake of O_2 (μl./30 min./flask)		
		T. rhodesiense	*T. gambiense*	*S. oncopelti*
DL-α-Glycerophosphate	Nil	20	32	22
	Cyanide	6	10	7
	Antimycin A	14	19	6
Succinate	Nil	37	27	51
	Cyanide	7	5	14
	Antimycin A	12	8	15
DPNH	Nil	87	55	109
	Cyanide	87	73	135
	Antimycin A	95	58	106

Table 6. *Dehydrogenase activity in freeze-dried preparations*

Reaction mixtures contained freeze-dried preparation (about 200 μg. of N for blood-stream forms and about 3 mg. of N for culture forms), tris buffer, pH 7·6 (0·14 M), phenazine methosulphate (4·6 mM), 8-hydroxyquinoline (mM) and either DL-α-glycerophosphate or succinate (33 mM) in a total volume of 3 ml. Preparations of culture forms (*C*) and blood-stream forms (*B*) were assayed at 30° and 37° respectively. The reaction was started by tipping phenazine methosulphate from the side arm and the oxygen uptake was recorded over 15 min. All results are corrected for an endogenous activity which in no case exceeded 2 μl. of O_2/10 min./mg. of N. – Signifies value not determined.

	Uptake of O_2 (μl./10 min./mg. of N)		
Species	(*a*) DL-α-Glycero-phosphate	(*b*) Succinate	Ratio (*b*):(*a*)
T. rhodesiense (*B*)	410	15	1:27
T. gambiense (*B*)	430	–	–
T. evansi (*B*)	414	–	–
T. rhodesiense (*C*)	21	39	1:0·5
T. gambiense (*C*)	18	32	1:0·5
S. oncopelti (*C*)	10	19	1:0·5

DISCUSSION

The present experiments establish that trypanosomes and other related protozoa contain different types of respiratory systems in particulate subcellular fractions, a conclusion in agreement with previous studies (Brand, 1951; Ryley, 1956; Fulton & Spooner, 1959), which have been mainly concerned with the spectroscopic examination of intact cell suspensions and with the effects of inhibitors on their respiration.

The high activity and substrate specificity of the L-α-glycerophosphate-oxidase system (1) appears to be a characteristic property of the blood-stream forms of the *brucei* group, which includes many trypanosomes of veterinary and medical importance. A cytoplasmic DPN-linked L-α-glycerophosphate dehydrogenase (2) is also present in these cells and the results given in Table 2 show that the coupled functioning of the reactions catalysed by these enzymes results in the oxidation of DPNH by indirect hydrogen transfer to the respiratory chain via L-α-glycerophosphate. In this manner,

$$\text{L-α-Glycerophosphate} + \tfrac{1}{2}O_2 \rightarrow \text{dihydroxyacetone phosphate} + H_2O \quad (1)$$

$$\text{Dihydroxyacetone phosphate} + DPNH + H^+ \rightarrow \text{L-α-glycerophosphate} + DPN^+ \quad (2)$$

$$\text{Sum:} \quad DPNH + H^+ + \tfrac{1}{2}O_2 \rightarrow DPN^+ + H_2O$$

it is possible to account for the uptake of oxygen observed when DPNH was added to crushed cell-free suspensions of *T. rhodesiense* (Fulton & Spooner, 1959).

The significance of these coupled reactions to the oxidative catabolism of glucose by *T. rhodesiense* has been discussed by Grant & Sargent (1960). The other species of the *brucei* group used in the present experiments catabolize glucose by the Embden–Meyerhof glycolytic pathway to pyruvate and a trace of glycerol (Ryley, 1956; Grant & Fulton, 1957), the rates of respiration being similar and in the range 166–194 μl. of oxygen/hr./10^8 cells (Brand, Tobie & Mehlman, 1950). These rates are about double those observed for the isolated L-α-glycerophosphate oxidase, and, although the former were calculated from studies involving both normal and infected blood, it seems probable that the oxidase was partially inactivated during the isolation procedure. In agreement with this view, it has been found that the activity of the particulate L-α-glycerophosphate dehydrogenase is about double that of the corresponding oxidase. Previous experiments indicated that the particulate dehydrogenase is a component of the oxidase system (Grant & Sargent, 1961) and for these reasons it is suggested that the major part of the respiration of this group of trypanosomes can be accounted for by the L-α-glycerophosphate-oxidase system.

Particulate preparations obtained from all strains were capable of oxidizing DL-α-glycerophosphate to a greater or less extent and two different respiratory systems could be distinguished. The L-α-glycerophosphate oxidase present in blood-stream forms of the *brucei* group was insensitive to both cyanide and antimycin A, a result in agreement with the repeated observation that trypanosomes of this group do not contain detectable cytochrome pigments (Brand, 1951; Ryley, 1956; Fulton & Spooner, 1959). In contrast, succinoxidase and α-glycerophosphate-oxidase activities in the culture forms of *T. rhodesiense*, *T. gambiense* and *S. oncopelti*, and in the blood-stream form of *T. lewisi* were inhibited by cyanide and to a lesser

extent by antimycin A. These inhibitors are believed to function specifically at single loci in complex respiratory systems; cyanide on cytochrome oxidase (Keilin & Hartree, 1939) and antimycin A at a locus between cytochrome *b* and *c* (Thorn, 1956). In these experiments, the degree of inhibition caused by each reagent was independent of the substrate used, so that the individual dehydrogenases may be structurally and functionally linked to a common cytochrome chain as has been shown in brain mitochondria by Ringler & Singer (1959).

In addition to the indirect system present in blood-stream forms of the *brucei* group, two other respiratory systems may also be distinguished for hydrogen transfer from DPNH to oxygen as the final acceptor. Thus, DPNH-oxidase activity in preparations of *T. lewisi* was sensitive to cyanide and antimycin A, whereas the system in preparations obtained from the culture forms of *T. rhodesiense*, *T. gambiense* and *S. oncopelti* was insensitive to these reagents. This latter result was unexpected since the intact cells contain cytochrome pigments and their respiration is readily inhibited by cyanide (Ryley, 1955; Fulton & Spooner, 1959). A functional cytochrome system need not, however, be precluded since suprarenal microsomes (Kersten, Kersten & Staudinger, 1958) and mitochondria of either the mid-gut of the silkworm (Pappenheimer & Williams, 1954), or the spadix of *Arum maculatum* (Bendall, 1958) can oxidize DPNH by a system in which a member of the *b* group of cytochromes can act as a terminal oxidase which is insensitive to both cyanide and antimycin A. Alternatively, the oxidation of DPNH by preparations of these culture forms may be effected by an aerobic dehydrogenase similar to that present in *Streptococcus faecalis* (Dolin, 1956).

Among the differences which have been observed between the respiratory systems of the various trypanosomes, perhaps the most interesting is the difference between the type of oxidase and the magnitude of the dehydrogenase activity in the two forms of *T. rhodesiense*. It is usually accepted that the culture form of this trypanosome corresponds to the mid-gut form which is the first of the three known forms to develop in the vector tsetse fly (Trager, 1959). The transition from a warm-blooded mammalian host to an invertebrate involves marked changes in the environment of the flagellate and would include that of temperature and oxygen tension. The latter change might induce an alteration in the type of respiratory system being utilized by the parasite, and an induction of this type has, in fact, been shown to occur in cultures of *Pseudomonas fluorescens* grown under conditions of varying oxygen concentration (Lenhoff, Nicholas & Kaplan, 1956).

SUMMARY

1. Cell-free preparations from blood-stream trypanosomes of the *brucei* group contain a cytoplasmic L-α-glycerophosphate dehydrogenase and a particulate hydrogen-transport system whose rate of reaction with L-α-glycerophosphate was about 20 times that of reduced pyridine nucleotide or succinate. The reaction of the L-α-glycerophosphate-oxidase system with oxygen was insensitive to both cyanide (0·5 mM) and antimycin A (5 μg./ml.).

2. Cell-free preparations from blood-stream forms of *Trypanosoma congolense*, *Trypanosoma lewisi* and *Trypanosoma vivax* oxidize α-glycerophosphate, succinate and reduced diphosphopyridine nucleotide at about the same rate. The oxidation of these substrates by preparations of *Trypanosoma lewisi* was inhibited to about the same extent by cyanide (0·5 mM). Antimycin A (5 μg./ml.) was also an inhibitor.

3. The ratio of the rates of oxygen uptake by cell-free preparations of the culture forms of *Trypanosoma rhodesiense*, *Trypanosoma gambiense*, *Trypanosoma cruzi*, *Strigomonas oncopelti* and *Leishmania donovani* was about 1:2:5 for α-glycerophosphate, succinate and reduced diphosphopyridine nucleotide respectively. α-Glycerophosphate- and succinic-oxidase activity in the first three of these species was inhibited by cyanide and antimycin A. In contrast, the oxidation of reduced diphosphopyridine nucleotide was slightly stimulated by these reagents.

4. L-α-Glycerophosphate- and succinic-dehydrogenase activities were measured with phenazine methosulphate as electron acceptor in particulate preparations obtained from freeze-dried samples of both blood-stream and culture forms.

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L-α-Glycerophosphate Dehydrogenase, a Component of an Oxidase System in *Trypanosoma rhodesiense*

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The oxidation of α-glycerophosphate to dihydroxyacetone phosphate with concomitant hydrogen transfer to molecular oxygen may be catalysed by various enzymes or enzyme systems which do not contain pyridine nucleotides. An aerobic α-glycerophosphate dehydrogenase from *Streptococcus faecalis* has been identified as an autooxidizable flavoprotein (Jacobs & Van Demark, 1960) and an enzyme of similar properties has also been found in *Escherichia coli* (Strittmatter, 1960). Rabbit muscle (Green, 1936), mitochondria of pig brain (Ringler & Singer, 1959), sarcosomes of insect flight muscle (Estabrook & Sacktor, 1958; Klingenberg & Bücher, 1959) and mitochondria of the mid-gut of the silkworm (Ito & Horie, 1959) all contain an anaerobic α-glycerophosphate dehydrogenase that is structurally and functionally linked to a respiratory chain containing cytochrome pigments. The complete system catalyses the reaction:

$$\alpha\text{-Glycerophosphate} + \tfrac{1}{2}O_2 \rightarrow \text{dihydroxyacetone phosphate} + H_2O.$$

Another oxidase system which catalyses the same reaction is present in cell-free preparations of blood-stream trypanosomes of the *brucei* group (Grant & Sargent, 1960*a*; Grant, Sargent & Ryley, 1961) but differs from that of mammalian and insect tissue in that the reaction with oxygen is not mediated by cytochrome pigments. Moreover, preparations of these protozoa exhibit no appreciable oxidase activity towards either succinate or reduced pyridine nucleotide as substrate.

The present work is concerned with some properties of an anaerobic L-α-glycerophosphate dehydrogenase, which is the first component of the trypanosomal oxidase system to be identified.

Some of these results have been reported by Grant & Sargent (1960*b*).

EXPERIMENTAL

Organism. The Liverpool strain of *Trypanosoma rhodesiense*, its maintenance in rats and the isolation of these cells from infected rat blood have been described by Grant & Fulton (1957). Microscopic examination was made on smears treated with Giemsa stain and the preparation was retained for experimental use if the content of platelets and red and white cells was negligible.

Preparation of cell-free extracts. Cell-free particulate preparations of *T. rhodesiense* containing L-α-glycerophosphate oxidase activity were obtained as described by Grant & Sargent (1960*a*).

In most experiments, the particulate material was dried from the frozen state immediately after preparation in a centrifugal freeze-drier (Edwards High Vacuum Ltd., Sussex) with P_2O_5 as desiccant. The dried material was stored *in vacuo* at 2°. Immediately before use, it was resuspended in distilled water (2 mg./ml.) with the aid of either an all-glass homogenizer of the Potter–Elvehjem type (Loughborough Glass Co., Loughborough, Leics.), or a 60 w ultrasonic vibrator of the magnetostrictor type with a

stainless-steel resonating probe of end ratio 9:1 (Measuring and Scientific Equipment Ltd., London, S.W. 1).

Measurement of enzyme activity. Consumption of O_2 was measured in a conventional Warburg apparatus at 37° in air. The total fluid volume added to each flask was 3·0 ml. In all cases, flasks were temperature-equilibrated for 10 min. before the start of the reaction. Cell-free preparations did not produce detectable CO_2 and, accordingly, KOH was not added to the centre well in these experiments.

The enzyme reduction of 2:6-dichlorophenol-indophenol was measured by following the extinction changes at 600 mμ of the reaction mixture contained in a 1 cm.² quartz cell fused to a standard Thunberg attachment (Thermal Syndicate Ltd., Wallsend, Northumberland). The cell, containing the reaction mixture with enzyme suspension in the stopper of the Thunberg tube, was evacuated four times and flushed each time either with O_2-free N_2 or with air. The compartment of the spectrophotometer containing the cell was covered with a light-tight box. Time was allowed for equilibration of temperature (about 20°), the enzyme was then added from the stopper and readings were commenced 30 sec. later. The amount of dye reduced was calculated from the molar extinction coefficient: $\epsilon\ 21 \times 10^3$, at 600 mμ (Savage, 1957).

Extinction changes in reactions involving DPN were measured at 340 mμ in a 1 cm.² quartz cell.

All optical measurements were made in a Hilger Uvispek spectrophotometer.

Analytical methods. Total N was determined by the micro-Kjeldahl method, the NH_3 produced being distilled into a boric acid solution containing methyl red–bromocresol green indicator.

Total phosphorus was determined as orthophosphate after digestion with $HClO_4$ (sp.gr. 1·7) at 160°, by the method of Berenblum & Chain (1938) with the micro-modification of Long (1943).

α-Glycerophosphate was determined as orthophosphate after periodate oxidation followed by acid hydrolysis (Burmaster, 1946).

Total triose phosphate was determined as alkali-labile phosphate (Meyerhof & Lohmann, 1934).

In one group of experiments (Table 2) both triose phosphate and α-glycerophosphate were determined in the same sample as described by Grant & Sargent (1960*a*).

Materials. DL- and L-α-Glycerophosphate were synthesized and solutions containing the sodium salts were stored as described by Grant & Sargent (1960*a*).

2:6-Dichlorophenol-indophenol was purified by the method of Savage (1957). 8-Hydroxyquinoline was recrystallized twice from aqueous 50% (v/v) methanol. Phenazine methosulphate, obtained from the Aldrich Chemical Corp. Inc., Milwaukee, Wis., U.S.A., was recrystallized twice from ethanol.

The following materials were used without additional purification: oxidized glutathione, DPN, DPNH, tris, mammalian cytochrome *c*, crystalline catalase and crystalline DPN-linked L-α-glycerophosphate dehydrogenase were obtained from C. F. Boehringer und Soehne GmbH., Mannheim, Germany; the dried venom of the cottonmouth mocassin (*Agkistrodon piscivorus piscivorus*) was obtained from Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A., and was used as a source of phospholipase A (cf. Long & Penny, 1957).

Pure H_2O_2 containing no added stabilizing agent was a gift from Laporte Chemicals Ltd., Luton, Beds. Melarsen oxide [*p*-(2:4-diamino-1:3:5-triazin-6-ylaminophenylarsenoxide] was a gift from May and Baker Ltd., Dagenham, Essex. Propane-1:2-diol 1-phosphate was a gift from Dr E. Huff and had been prepared by the method of Huff & Rudney (1959).

RESULTS

The activity of the L-α-glycerophosphate oxidase present in particulate preparations of *T. rhodesiense* was completely lost after freeze-drying or by treatment with acetone at −15°. The residual material, however, was capable of oxidizing L-α-glycerophosphate to dihydroxyacetone phosphate in the presence of a suitable electron acceptor such as phenazine methosulphate or 2:6-dichlorophenol-indophenol (Tables 1 and 2). This activity was essentially unchanged after 1 year's storage at 2°, and will be subsequently referred to as L-α-glycerophosphate dehydrogenase.

Freeze-dried preparations were suspended in

Table 1. *Activity of* L-α-*glycerophosphate dehydrogenase with various electron acceptors*

Reaction mixtures contained tris buffer, pH 7·6 (0·14 M), DL-α-glycerophosphate (33 mM), freeze-dried enzyme preparation and the other additions shown below in a total volume of 3·0 ml. The reduction of 2:6-dichlorophenol-indophenol and cytochrome *c* were measured spectrophotometrically at 600 and 550 mμ respectively as described in the Experimental section. Manometric procedures were used for other assays. In all cases the reaction was started by the addition of electron acceptor and the activity was calculated from either the uptake of O_2 in 15 min., or the degree of reduction of the acceptor in 3 min.

Addition	Enzyme added (mg. of N)	Activity (μmoles of substrate utilized/10 min./mg. of N)
Phenazine methosulphate (4·6 mM) and 8-hydroxyquinoline (mM)	0·14	21·5
2:6-Dichlorophenol-indophenol (0·13 μM)	0·05	3·0
Methylene blue (3·5 mM)	1·00	1·5
Brilliant cresyl blue (4·2 mM)	1·00	Nil
Cytochrome c (1·0 mg.) and cyanide (mM)	1·00	Nil

0·02 M-sodium pyrophosphate buffer, pH 8·6 (5 mg./ml.), and subjected to ultrasonic vibration for 6 min. Essentially all the L-α-glycerophosphate-dehydrogenase activity was present in the residue after centrifuging at 104 000*g* for 60 min., the specific activity (about 1900 μl. of oxygen utilized/60 min./mg. dry wt.) being about four times that of the starting material. Further purification of this enzyme was difficult because no procedure was found which would release the active enzyme from the particulate material; treatment with salt solutions of various pH values and ionic strengths, trypsin, phospholipase A, butan-1-ol and deoxycholate was not effective.

Assay of L-α-*glycerophosphate dehydrogenase with phenazine methosulphate*

Kinetic aspects. The following results characterize the enzyme and define the optimum conditions for its assay.

The effect of pH on the enzyme activity in both phosphate and tris buffers in the range pH 6–9 is shown in Fig. 1. The pH optima were about pH 7·2 and 8·2 respectively, the enzyme being appreciably more active when tris buffer was used in the assay system. This result is in marked contrast with the α-glycerophosphate dehydrogenase in the mitochondria of pig brain, which has a maximum activity in phosphate buffer with the same electron acceptor (Ringler & Singer, 1959).

Enzyme activity was determined with different concentrations of substrate and electron acceptor (Fig. 2). From these results the K_m values for substrate and acceptor were calculated by the reciprocal-plot method (Lineweaver & Burk, 1934) to be 9·0 and 0·34 mM respectively.

With saturating concentrations of substrate (40 mM) and electron acceptor (4·6 mM), the activity of the enzyme was proportional to the amount of enzyme added up to about 300 μg. of N. When greater amounts of enzyme were used the rate was limited by the diffusion of oxygen into the reaction medium, and, for this reason, about 140 μg. of N were used in subsequent determinations of enzyme activity (140 μg. of N ≡ 1·0 mg. dry wt. of freeze-dried material).

In this assay system the addition of 8-hydroxyquinoline was essential for the maximum reaction rate (Fig. 3), an effect which was independent of the concentration of 8-hydroxyquinoline added in the range 0·6–3·0 mM.

Stoicheiometric relationships and substrate specificity. Glycerol, β-glycerophosphate and propane-1:2-diol 1-phosphate neither served as substrates for the enzyme nor, when added singly in equimolar amounts with L-α-glycerophosphate, had any effect on the oxidation of the latter.

The quantitative relationship between reactants

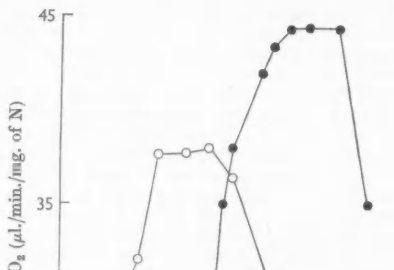

Fig. 1. Effect of pH on activity. Reaction mixtures contained freeze-dried preparation (140 μg. of N), buffer (0·14 M), DL-α-glycerophosphate (33 mM), phenazine methosulphate (4·6 mM) and 8-hydroxyquinoline (mM) in a total volume of 3·0 ml. Reaction was started by addition of phenazine methosulphate from the side arm and readings were recorded over a period of 10 min. The pH of the flask contents was subsequently determined by glass electrode at 20°. ○, Phosphate buffer; ●, tris buffer.

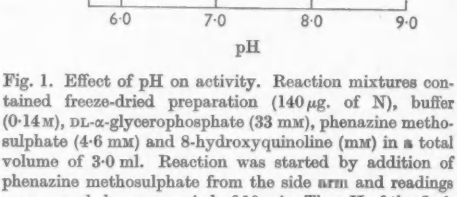

Fig. 2. Lineweaver–Burk plots {reciprocal of the initial rate, V (μl. of O_2 utilized/10 min./flask), against the reciprocal of substrate concentration, [S] (mM-substrate), and electron acceptor concentration, [D] (mM-acceptor)} for L-α-glycerophosphate dehydrogenase. Reaction mixtures contained freeze-dried preparation (140 μg. of N), tris buffer, pH 7·6 (0·14 M), 8-hydroxyquinoline (mM), DL-α-glycerophosphate and phenazine methosulphate. ●, DL-α-Glycerophosphate (33 mM) with varied concentrations of phenazine methosulphate; ○, phenazine methosulphate (4·6 mM) with varied concentrations of DL-α-glycerophosphate. Reaction was started by the addition of phenazine methosulphate from the side arm and readings were recorded over a period of 10 min.

and products was determined by analysis of the reaction mixture at the beginning and end of each experiment. In the presence of 8-hydroxyquinoline, the results (Expt. 1, Table 2) are consistent with the reaction:

$$\text{L-α-Glycerophosphate} + O_2 \rightarrow \text{triose phosphate} + H_2O_2.$$

This result is in agreement with that given in Expt. 2, Table 2, in which the addition of an excess of catalase reduced the uptake of oxygen in the presence of 8-hydroxyquinoline to 1 g.atom/mole of substrate utilized.

Dihydroxyacetone phosphate was identified as the triose phosphate present in the reaction mixture. This was shown by the decrease in extinction at 340 mμ of a solution containing DPNH, crystalline DPN-linked L-α-glycerophosphate dehydrogenase and a portion of the reaction mixture obtained at the end of the experiments after removal of protein and phenazine methosulphate. This latter compound catalysed a non-enzymic oxidation of DPNH (Du Buy & Showacre, 1959) and was quantitatively absorbed by percolation of the protein-free reaction mixture through columns (5 cm. × 1 cm.) of Zeo-Karb 225 (H^+ form). The enzyme preparations did not contain detectable triose phosphate isomerase (cf. Grant & Sargent, 1960*a*) and for this reason it was concluded that dihydroxyacetone phosphate and not glyceraldehyde 3-phosphate was the product of the dehydrogenase reaction.

Effect of thiol group reagents. The activity of L-α-glycerophosphate dehydrogenase, although unaffected by thiol-alkylating agents such as iodoacetic acid and iodoacetamide, was sensitive to N-ethylmaleimide. *p*-Chloromercuribenzoate, organic tervalent arsenicals, various metal ions (Table 3) and hydrogen peroxide (Table 4), all of which are known to affect thiol groups, inhibited enzyme activity. The effect of *p*-chloromercuribenzoate was particularly marked (Table 3, Fig. 5) and this inhibition could be completely reversed by incubating the treated enzyme with glutathione (mM) at 37° for 10 min.

An enzyme preparation was treated either with potassium cyanide (10 mM) for 12 hr. at 18° or with oxidized glutathione (50 mM) for 6 hr. at 37° exactly as detailed by Keilin & King (1960) in a study of mammalian succinic dehydrogenase. In neither case, however, did the treatment result in any significant decrease in L-α-glycerophosphate-dehydrogenase activity with phenazine methosulphate as electron acceptor.

Effect of metal-chelating and other agents. Chelating agents had no effect on the enzyme activity when included in the assay system involving 2:6-dichlorophenol-indophenol. In contrast, when phenazine methosulphate was used as electron acceptor, the uptake of oxygen ceased about 6 min. after the addition of acceptor unless either catalase or a chelating agent, preferably 8-hydroxyquinoline, was present (Fig. 3). In the presence of 8-hydroxyquinoline the rate of oxygen uptake was double that observed when both catalase and 8-hydroxyquinoline were present, a result in agreement with the stoicheiometry of these reactions (Table 2).

The inhibitory effects of hydrogen peroxide were greatly enhanced by the presence of aqueous extracts of enzyme ash or added metal ions (Table 4). These effects were relieved by 8-hydroxyquinoline and to a lesser extent by cyanide, which

Table 2. *Quantitative relationships between substrate and product of the* L-α-*glycerophosphate-dehydrogenase reaction*

Reaction mixtures contained freeze-dried preparation (140 μg. of N), tris buffer, pH 7·6 (0·14 M), L-α-glycerophosphate (5·5 mM), phenazine methosulphate (4·6 mM) and either 8-hydroxyquinoline (mM) in Expt. 1, or catalase (5 μM) and 8-hydroxyquinoline (mM) in Expt. 2, in a total volume of 3·0 ml. Reaction was started in each case by addition of phenazine methosulphate from the side arm and stopped after 12 min. by rapid cooling to 0° with simultaneous addition of 0·5 ml. of 30% (w/v) trichloroacetic acid.

Expt. no.	Uptake of O_2 (μmoles)	Triose phosphate formed (μmoles)	L-α-Glycerophosphate used (μmoles)
1	4·4	4·2	4·8
2	1·7	3·2	3·4

Table 3. *Effect of inhibitors on* L-α-*glycerophosphate-dehydrogenase activity*

The main compartment of each flask contained freeze-dried enzyme preparation (140 μg. of N), tris buffer, pH 7·6 (0·14 M), and the additions shown below in a total volume of 2·0 ml. The side arm contained DL-α-glycerophosphate (33 mM), 8-hydroxyquinoline (mM) and phenazine methosulphate (4·6 mM) in a total volume of 1·0 ml. After equilibration for 10 min. at 37° the reaction was started by addition of the side-arm contents.

Addition	Final concn.	Uptake of O_2 (μl./15 min.)
Nil	—	108
3-Amino-4-hydroxyphenyl-arsenoxide	10 μM	67
Melarsen oxide	10 μM	67
N-Ethylmaleimide	0·1 mM	59
p-Chloromercuribenzoate	1 μM	58
$CuSO_4$	50 μM	39
$ZnSO_4$	0·5 mM	53
$CdSO_4$	0·5 mM	103
Iodoacetate* or iodoacetamide*	1 mM	108
Sodium arsenite	0·5 mM	108

* Equilibration for either 10 or 30 min. with enzyme before assay.

Table 4. *Inhibition by oxidizing and mercaptide-forming reagents and the protective effect of other components in the reaction mixture*

Conditions for these experiments are given in Table 3, except that the enzyme was treated as shown below for 15 min. at 37° and the reaction was started by tipping the remaining components of the reaction mixture from the side arm.

Expt. no.	Treatment	Uptake of O_2 (μl./15 min.)
1	Nil	94
	Phenazine methosulphate (13·8 μmoles)	73
	Enzyme mixed with 8-hydroxyquinoline (3 μmoles) and treated with phenazine methosulphate	75
2	Nil	101
	Unstabilized H_2O_2 (0·4 μmole)	43
	Enzyme mixed with 8-hydroxyquinoline (3 μmoles) and treated with H_2O_2	98
	Enzyme mixed with cyanide (3 μmoles) and treated with H_2O_2	78
	Enzyme mixed with substrate (100 μmoles) and treated with H_2O_2	67
	Enzyme mixed with phenazine methosulphate (13·8 μmoles) and treated with H_2O_2	56
3	Nil	112
	Cu^{2+} ions (0·05 μmole)	104
	Cu^{2+} ions and H_2O_2 (0·4 μmole)	21
	Enzyme mixed with 8-hydroxyquinoline and treated with Cu^{2+} ions and H_2O_2	105
4	Nil	90
	Extract of enzyme ash (equivalent to 300 μg. of enzyme N)	90
	Extract of enzyme ash and H_2O_2 (0·4 μmole)	27
	Enzyme mixed with 8-hydroxyquinoline (3 μmoles) and treated with extract of enzyme ash and H_2O_2	86
5	Nil	104
	p-Chloromercuribenzoate (0·01 μmole)	25
	Enzyme mixed with substrate and treated with *p*-chloromercuribenzoate	40
	Enzyme mixed with phenazine methosulphate and treated with *p*-chloromercuribenzoate	59

would indicate that some essential group on the enzyme is sensitive to a metal-catalysed oxidation by hydrogen peroxide. The concentration of hydrogen peroxide added in these experiments was of the same order as that calculated to be present in the assay system after about 6 min., so that the protective effect of 8-hydroxyquinoline, and possibly also that of cyanide, in this system can be attributed to their metal-chelating properties.

Relationship between L-α-*glycerophosphate dehydrogenase and oxidase*

The effect of aerobic and anaerobic conditions on the rate of reduction of 2:6-dichlorophenol-indophenol by a fresh cell-free preparation containing L-α-glycerophosphate-oxidase activity was compared with that of a freeze-dried sample of the same preparation which contained only L-α-glycerophosphate-dehydrogenase activity (Fig. 4). No significant difference was found between the activity of the fresh preparation and the freeze-dried preparation under anaerobic conditions, thus showing that the L-α-glycerophosphate dehydrogenase originally present had unimpaired activity after desiccation. Nevertheless, the rate of dye reduction by the fresh preparation under aerobic conditions was about 70 % of that found anaerobically. This difference would suggest that the dehydrogenase is a component of the oxidase system and can transfer electrons either to the terminal part of the respiratory chain or to a suitable acceptor such as 2:6-dichlorophenol-indophenol: the proportion of electrons transferred to either acceptor would be determined by the experimental conditions. These experiments have not been pursued but are in general agreement with those given below.

A comparison was made of the effects of *p*-chloromercuribenzoate on oxidase activity of fresh and aged portions of the same preparation, and of dehydrogenase activity with phenazine methosulphate as acceptor in the same material after freeze-drying. Under these directly comparable conditions (Fig. 5) the dehydrogenase had twice the activity (μmoles of substrate utilized/min./mg. of N) of the freshly prepared oxidase. The dehydrogenase was progressively inhibited by increasing concentrations of *p*-chloromercuribenzoate, whereas the activity of the oxidase was essentially unchanged until a concentration of

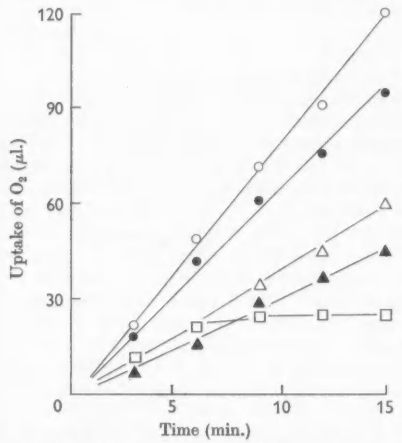

Fig. 3. Effect of metal-binding and other agents. Reaction mixtures contained freeze-dried preparation (140 μg. of N), tris buffer, pH 7·6 (0·14 M), DL-α-glycerophosphate (33 mM), phenazine methosulphate (4·6 mM) and the following additions: ○, 8-hydroxyquinoline (mM); ●, KCN (mM) or ethylenediaminetetra-acetic acid (mM); △, catalase (2·5 mg.) and 8-hydroxyquinoline (mM); ▲, catalase (2·5 mg.); □, no addition. The reaction was started in all cases by the addition of phenazine methosulphate from the side arm.

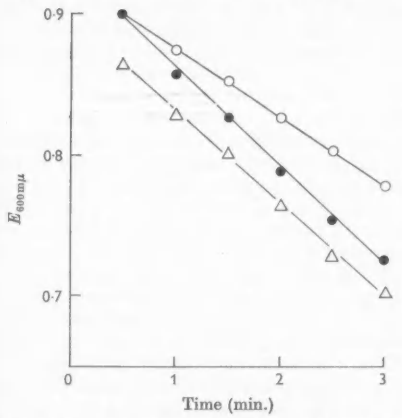

Fig. 4. Effect of aerobic and anaerobic conditions on fresh and freeze-dried enzyme preparations with 2:6-dichlorophenol-indophenol as electron acceptor. Details of these experiments are given in Table 1 and in the Experimental section, except that in all experiments the same amount of enzyme preparation (30 μg. of N) was used. ○, Freshly prepared enzyme under aerobic conditions; ●, freshly prepared enzyme under anaerobic conditions; △, freeze-dried sample of fresh preparation under either aerobic or anaerobic conditions.

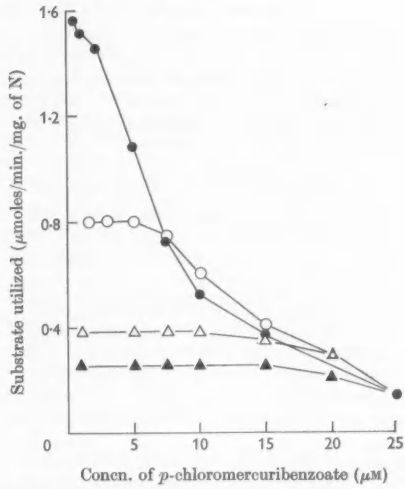

Fig. 5. Comparison of the effects of *p*-chloromercuribenzoate on the activities of L-α-glycerophosphate oxidase and dehydrogenase. Oxidase activity: the enzyme preparation was incubated at 37° for 10 min. with tris buffer, pH 7·6 (0·14 M), and *p*-chloromercuribenzoate as shown below in a total volume of 2·0 ml. The reaction was started by the addition of DL-α-glycerophosphate (1 ml.; 33 mM) from the side arm and the initial rate of the reaction was measured over a period of 15 min. Dehydrogenase activity: reaction mixtures and procedure were as described in Table 3. ○, Oxidase activity in the fresh preparation (275 μg. of N); △, oxidase activity in the same preparation after 4 hr. at 0°; ▲, oxidase activity in the same preparation after 10 hr. at 0°; ●, dehydrogenase activity in material (275 μg. of N) obtained by freeze-drying the fresh preparation immediately, or after 10 hr. at 0°.

inhibitor was added which reduced dehydrogenase activity to about the value that corresponded to the activity of the oxidase. Thereafter higher concentrations of *p*-chloromercurbenzoate resulted in an approximately parallel decrease in activity of both oxidase and dehydrogenase.

The activity of the freshly prepared oxidase was usually about half that of the dehydrogenase. This could indicate that the dehydrogenase is not the rate-limiting reaction of the oxidase system. However, no special precautions were taken to protect the latter during its preparation and, as the activity rapidly decreased on standing at 2° (Fig. 5), it was probable that all oxidase preparations contained some inactivated enzyme in amounts which progressively increased with time after the initial rupture of the intact cell. In contrast, dehydrogenase activity in these preparations measured after the sample had been freeze-dried was essentially the same as that of the intact

cells which were freeze-dried without prior treatment. For these reasons, any consideration of the results given in Fig. 5 must take into account the uptake of *p*-chloromercuribenzoate by inactivated enzyme when the oxidase activity of both fresh and aged samples was being measured.

DISCUSSION

The results show that particulate preparations of *T. rhodesiense* contain an anaerobic L-α-glycerophosphate dehydrogenase that is functionally linked to a terminal respiratory chain. This latter system does not involve cytochrome pigments (Grant & Sargent, 1960*a*), and, although the components have not been identified, the results given in Fig. 5 are consistent with the view that the thiol group, which is essential for the activity of the intact oxidase, is located in the dehydrogenase component.

Although insensitive to iodoacetamide, the marked inhibition of L-α-glycerophosphate-dehydrogenase activity by *N*-ethylmaleimide, tervalent organic arsenicals and *p*-chloromercuribenzoate would indicate that thiol groups have an essential role in the activity of the enzyme. 'Dithiol' groups do not appear to be involved since the enzyme, like that present in mammalian muscle (Hopkins & Morgan, 1938), was not affected by a prolonged incubation with oxidized glutathione. Cyanide was also ineffective in this respect. The L-α-glycerophosphate dehydrogenase studied in these experiments thus differs from succinic dehydrogenase of mammalian heart muscle in that the latter enzyme contains a 'dithiol' group that is inhibited by oxidized glutathione (Hopkins & Morgan, 1938; Singer, Kearney & Massey, 1957; Keilin & King, 1960) and a distinct and additional group that combines slowly and irreversibly with cyanide (Tsou, 1951; Giuditta & Singer, 1959; Keilin & King, 1960). An indication that two spatially adjacent thiol groups are not involved with the activity of L-α-glycerophosphate dehydrogenase is that the enzyme was insensitive to both arsenite and Cd^{2+} ions. At low concentrations these compounds are considered to be specific inhibitors of reactions involving such groups (Sanadi, Langley & White, 1959). Arsenite forms only a weak complex with monothiols, but forms a stable ring structure with dithiol compounds provided that the thiol groups are spatially adjacent (Stocken & Thompson, 1946).

The insensitivity of the L-α-glycerophosphate-oxidase system to concentrations of *p*-chloromercuribenzoate that markedly inhibited the activity of the dehydrogenase (Fig. 5) would suggest that the thiol group does not react so readily with the inhibitor when the dehydrogenase is functionally linked to the respiratory chain to form the intact oxidase system. All preparations of the oxidase contained some inactivated enzyme and the amount progressively increased with time; in contrast, the dehydrogenase activity measured with phenazine methosulphate as acceptor was essentially unchanged by storage. For these reasons, it had been concluded that the oxidase activity in both fresh and aged preparations would be unaffected by *p*-chloromercuribenzoate until the thiol groups in the dehydrogenase component of the inactivated enzyme had first been saturated with the inhibitor. Moreover, the reaction of the dehydrogenase with *p*-chloromercuribenzoate can be partly prevented by the prior addition of phenazine methosulphate (Table 4), so that the thiol group may be concerned with electron transfer to either the respiratory chain or phenazine methosulphate.

The maximum rate of enzyme activity was obtained with phenazine methosulphate as electron acceptor. The enzyme was appreciably more active in tris than in phosphate buffer and the partly purified particulate material in tris buffer, pH 7·6, catalysed a linear rate of oxygen uptake equivalent to a Q_{O_2} (μl. of O_2/mg. dry wt./hr.) of about 1900. Phenazine methosulphate is also the preferred electron acceptor for the assay of α-glycerophosphate dehydrogenase of brain mitochondria (Q_{O_2} 170–243 in phosphate buffer, pH 7·6; Ringler & Singer, 1959) and of other cytochrome-linked dehydrogenase (Singer, Kearney & Massey, 1957; Mapson & Breslow, 1958; Rendina & Singer, 1959), but with these enzymes inorganic phosphate was essential for maximum reaction rates.

The assay of anaerobic dehydrogenases with phenazine methosulphate as electron acceptor is complicated by the necessity to protect the enzyme from hydrogen peroxide formed during the autoxidation of the reduced acceptor. In the original description of the phenazine methosulphate assay procedure for succinic dehydrogenase (Kearney & Singer, 1956) it was found that cyanide and 8-hydroxyquinoline were the most effective protective agents. Cyanide was assumed to react with disulphide (–S·S–) groups formed by the action of hydrogen peroxide on adjacent thiol groups, the enzyme being active when one member of the pair was converted into a thiocyanate (–SCN) and the other into a free thiol (–SH) group. In the present experiments with an enzyme that apparently does not contain adjacent thiol groups, 8-hydroxyquinoline and cyanide to a lesser extent were effective protective agents in the assay procedure. Fig. 3 would indicate that they inhibit a metal-catalysed oxidation by hydrogen peroxide of an essential thiol group on the enzyme, and the protective effect can thus presumably be attributed to

the metal-chelating properties of these compounds.

The particulate nature of the anaerobic α-glycerophosphate dehydrogenase of mammalian tissue was noted by Green (1936), who first systematically studied this enzyme. Subsequently it was shown that the enzyme could be extracted from tissue preparations by bile salts (Tung, Anderson & Lardy, 1952), bile salts and digestion with trypsin (Ling, Wu, Ting & Tung, 1957), digitonin (Ringler & Singer, 1958) and by treatment with phospholipase A (Ringler & Singer, 1959). These and other procedures were not effective with the particulate enzyme studied here and the nature of the electron pathway between L-α-glycerophosphate and phenazine methosulphate cannot be determined until the active enzyme can be released from the particulate material and purified. Flavin–adenine dinucleotide has been identified in these particulate preparations (P. T. Grant & J. R. Sargent, unpublished observations), but whether this coenzyme is concerned with L-α-glycerophosphate-dehydrogenase activity remains to be established.

SUMMARY

1. An enzyme has been found in particulate preparations of *Trypanosoma rhodesiense* which catalyses the oxidation of L-α-glycerophosphate to dihydroxyacetone phosphate in the presence of either phenazine methosulphate or 2:6-dichlorophenol-indophenol as electron acceptors. The enzyme has been termed an anaerobic L-α-glycerophosphate dehydrogenase.

2. A partly purified particulate fraction under optimum conditions with phenazine methosulphate as electron acceptor catalysed an oxygen uptake which corresponded to a Q_{O_2} of about 1900.

3. In the phenazine methosulphate assay procedure, the optimum enzyme activity in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer was about pH 8. The values of K_m for substrate and electron acceptor were 9·0 and 0·34 mM respectively.

4. L-α-Glycerophosphate-dehydrogenase activity was inhibited by certain thiol-group reagents. Inhibition by *p*-chloromercuribenzoate could be reversed by glutathione. The enzyme was insensitive to arsenite and Cd^{2+} ions. Prolonged incubation with either oxidized glutathione or cyanide had no significant effect on enzyme activity.

5. Inhibition of enzyme activity by hydrogen peroxide was enhanced in a catalytic manner by either extracts of enzyme ash or Cu^{2+} ions. 8-Hydroxyquinoline almost completely protected the enzyme from these agents and this chelating agent was added as a routine in the assay procedure to protect the enzyme from hydrogen peroxide formed during the auto-oxidation of the reduced acceptor.

6. A comparison of the activities of both L-α-glycerophosphate oxidase and the corresponding dehydrogenase was made on portions of the same preparation. The effects of *p*-chloromercuribenzoate were also studied. The results are consistent with the view that the dehydrogenase is a component of the oxidase system and that the former contains the thiol group that is essential for the activity of the oxidase.

We are greatly indebted to Professor W. O. Kermack, F.R.S., for many helpful discussions during the course of this work. We thank the Medical Research Council and the Tsetse-Fly and Trypanosomiasis Committee of the Colonial Office for grants in aid of expenses and equipment. One of us (J.R.S.) wishes to thank the Carnegie Trust for the Universities of Scotland for a scholarship.

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The Lipid Composition of Rat-Liver-Cell Sap

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The fatty acid composition of the total lipids of each of the different compartments (nuclei, mitochondria, fluffy layer, microsomes, supernatant and fatty supernatant) of the rat-liver cell was determined by Getz & Bartley (1961). The fatty acid composition of the different lipid classes (neutral lipids, aminophosphatides and cholinephosphatides) within a particular compartment of the liver cell, the cell sap, is described in this study.

METHODS

The cell sap was prepared from 92 g. of rat-liver pulp (obtained from eight male rats of the Wistar strain, average age 8 months and average weight 400 g.). It was obtained as the supernatant fluid that separated after sedimenting the particles from a homogenate of the livers in 0·25M-sucrose solution. The nuclear fraction and the mitochondria were isolated by the procedure of Werkheiser & Bartley (1957) and the fluffy layer was retained and washed in the same way as the mitochondrial sediment. The microsomes were isolated from the combined supernatants of the mitochondrial and fluffy layer fraction by centrifuging for 60 min. at 30 000*g* in no. 20 rotor of the Spinco centrifuge. A clear supernatant was obtained with a layer of fatty material floating on the surface. The floating fatty material was removed with a Pasteur pipette and the fat on the sides of the centrifuge tubes removed by careful wiping with fat-free filter paper. The combined material ('floating fatty supernatant') was then extracted with 20 vol. of chloroform–methanol (2:1, v/v). The cell sap was removed from above the microsomes with a Pasteur pipette. To avoid the extraction of large volumes of cell sap with organic solvents, the lipids were first co-precipitated with protein, with trichloroacetic acid as described by Getz & Bartley (1961), and then extracted from the trichloroacetic acid precipitates with 20 vol. of chloroform–methanol (2:1, v/v).

Analysis of cell sap. One portion of the extract was used for the total lipid determination and another was saponified and the fatty acids liberated were esterified with diazomethane, in each case by procedures outlined by Getz & Bartley (1961). The remainder of the lipid extract was chromatographed on silicic acid (Mallinckrodt Chemical Works; mesh 100, prepared according to the method of Hirsch & Ahrens, 1958). The loading procedure and elution sequence were carried out as described by Getz, Bartley, Stirpe, Notton & Renshaw (1961) except that the triglycerides were eluted with 500 ml. of 10% ether in light petroleum (b.p. 60–80°) and the remaining neutral glycerides with 300 ml. of diethyl ether before the first phospholipid fraction was eluted with 100 ml. of chloroform–methanol (7:1, v/v). The elution sequence was as shown in Table 1. Each lipid fraction was subjected to appropriate analysis by methods described by Getz *et al.* (1961). In addition, the methyl esters of the fatty acids of each fraction were prepared by interesterification and microsublimation (Stoffel, Chu & Ahrens, 1959) and the mixtures of methyl esters so obtained were analysed by gas chromatography.

Preparation of lipid extracts for silicic acid chromatography. Insoluble material was deposited from the chloroform–methanol extract of the cell sap during storage and during concentration of the extract for putting on to the silicic acid column. The extract was therefore evaporated to dryness under N_2 and extracted with warm chloroform. The chloroform extract was filtered and the residue on the filter paper washed three times with chloroform. The combined chloroform extracts were evaporated to dryness, dissolved in a small amount of light petroleum (b.p. 60–80°) and applied to the silicic acid column. The residue filtered off from the chloroform solution was saponified and the fatty

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Table 1. *Elution sequence used to fractionate the lipids of the cell sap on silicic acid*

Fraction no.	Solvent	Volume (ml.)	Main lipid expected
1	1% Ether in light petroleum (60–80°)	350	Sterol esters
	4% Ether in light petroleum (60–80°)	60	
2	10% Ether in light petroleum (60–80°)	500	Triglycerides
3	100% Ether	300	Lower neutral glycerides
4	Chloroform–methanol (7:1, v/v)	100	Non-nitrogen phosphatide, serine phosphatide
5	Chloroform–methanol (4:1, v/v)	200	Ethanolamine phosphatide
6	Chloroform–methanol (3:2, v/v)	100	Serine and inositol phosphatide
7	Chloroform–methanol (3:2, v/v)	400	Lecithin
8	Chloroform–methanol (1:4, v/v)	200	Sphingomyelin and other lipids

Table 2. *Analysis of lipid fractions from cell sap of rat liver*

Results are expressed as μmoles/g. dry wt. of cell sap.

No. of fraction	Lipid esters in fraction	Total P in fraction	Ester/ phosphate	Total choline in fraction	Choline/ phosphate	Total amino N in fraction	Amino N/ phosphate	Methyl esters/ phosphate
1	20·2	0	—	—	—	—	—	—
2	19·6	0·03	—	—	—	—	—	—
3	4·8	0·04	—	—	—	—	—	—
4	8·2	1·6	5·1	0	—	1·3	0·8	2·3
5	1·7	1·4	1·3	0	—	1·6	1·1	2·0
6	3·5	2·8	1·2	0	—	1·3	0·46	1·8
7	8·7	5·6	1·6	5·6	1·0	0·4	0·07	1·9
8	1·8	2·8	0·6	0	—	0·1	0·035	0·7

Table 3. *Distribution of lipids within the cell sap compared with the mean distribution throughout the cell*

A is the

$$\frac{\text{Wt. of lipid fraction in cell sap}}{\text{Wt. of same lipid fraction in total homogenate}} \times 100.$$

B compares the amount of a lipid class/unit dry wt. of cell sap with the amount of the same lipid class in unit dry wt. of the whole cell. The dry weights were determined by the method of Werkheiser & Bartley (1957) as the trichloroacetic acid-insoluble material.

Fraction no. Normal lipid class	Percentage distribution of esters within lipid class	*A*	*B*
1	29·6	32·1	1·34
2+3	35·7	8·7	0·35
4	12·0	13·4	0·56
5	2·54	0·75	0·03
6	5·07	3·1	0·13
7	12·8	1·3	0·055
8	2·56	19·6	0·73
Total		4·8	0·33

acids liberated were esterified. Yields of 3·28 μmoles of fatty acid ester/g. dry wt. of cell soluble sap were obtained. The fatty acid composition of this material is reported in Table 4. The triglyceride fraction from the 'floating fatty supernatant' contained a large proportion of free fatty acids. These were removed by extracting the fraction with 0·5% potassium carbonate in 50% methanol. After esterification, their fatty acid composition was examined (Table 7).

Some difficulty was experienced in obtaining the theoretical ester:phosphorus ratio when the fatty acid esters were measured directly on the various phospholipid fractions. After saponification and methylation of the fatty acids, the analyses of the methyl esters recovered were much nearer those expected from the phosphorus content of the phospholipids.

RESULTS

The results of the silicic acid fractionation and of the analysis of the various fractions of the cell sap are shown in Table 2. More than half the total lipid was in the form of neutral lipid.

In Table 3 (column *A*) the distribution of the various lipid fractions in the cell sap, is related to their distribution in the whole liver, the data obtained for the lipid composition of the rat liver given by Getz *et al.* (1961) being used.

Whereas only about 4·8% of the total lipid esters of the liver are found in the cell sap 32·1% of the presumed sterol esters (fraction 1) and 20% of the presumed sphingomyelin (fraction 8) are found in this fraction. By contrast, only 1·3% of the lecithin (fraction 7) is present in the cell sap.

The concentration of each lipid ester in the cell sap can also be related to the concentration of the same ester in the whole liver. This has been done in Table 3 (column *B*), concentrations being

Table 4. *Fatty acid composition of the cell sap of rat liver*

Values are given as percentages of the total methyl esters calculated from the gas chromatogram. The area of each peak was measured and expressed as a percentage of the total area under all the peaks.

br, branched chain; h.br, highly branched; uns, unsaturated.

Number of double bonds is given, e.g. as 18-1. This designates a fatty acid 18 carbon atoms long with one double bond; the position of the double bond is not given.

	Nominal lipid class								
Fatty acid	Sterol esters	Triglycerides	Lower neutral glycerides	Fraction eluted with chloroform–methanol (7:1)	Ethanolamine phosphatide	Serine phosphatides + inositides	Lecithin	Sphingolipid	Chloroform-insoluble fraction
*11-br	—	—	—	—	—	—	—	—	0·36
12-br	0·05	—	—	—	—	—	—	—	—
12-0	0·12	0·22	0·17	0·19	0·05	—	0·05	—	0·21
13-0	—	—	—	0·04	—	—	—	—	—
14-1	—	0·25	—	—	—	—	—	—	0·07
*14-h.br	0·04	0·21	—	0·30	0·07	0·04	—	—	—
14-0	0·87	0·97	1·06	0·54	0·25	0·13	0·55	0·34	0·73
*15-h.br	—	—	1·52	—	0·09	0·03	—	—	—
*15-h.br	—	—	0·38	0·56	—	—	—	—	—
*15-br	—	—	—	0·08	—	—	—	—	—
15-br	0·20	0·15	0·25	0·08	0·09	—	—	—	—
15-0	0·54	0·56	0·40	0·36	0·31	0·19	0·67	0·40	0·36
*16-h.br	—	—	—	—	—	0·06	—	—	—
*16-h.br	0·41	—	—	0·54	0·07	—	—	—	—
16-1	2·4	2·5	2·1	0·64	0·92	0·61	1·5	1·23	1·9
16-0	27·0	26·9	21·4	18·1	22·7	15·8	25·2	19·6	21·8
*17-h.br	—	—	—	—	0·06	—	—	—	—
17-br	0·43	0·42	0·53	0·34	0·41	0·36	0·38	0·31	—
17-0	0·63	0·69	0·31	0·82	0·90	0·76	0·99	0·64	0·56
*17-uns	—	—	—	0·30	0·36	0·39	0·28	—	—
*18-h.br	0·07	0·39	—	0·34	—	0·06	—	—	—
18-0	15·0	10·0	13·9	26·8	22·0	26·4	18·7	16·4	14·8
18-1	20·6	23·1	18·2	10·4	11·5	8·3	13·4	12·6	12·9
18-2	21·2	21·1	20·2	8·9	14·2	8·9	21·0	19·6	18·2
*18-2 or 3	—	—	1·25	—	—	—	—	—	—
*19-br	—	—	—	0·27	0·33	0·18	—	—	—
20-0	—	—	0·66	—	—	—	—	—	—
20-1	—	0·85	—	—	0·22	0·42	—	0·65	—
20-2	—	0·19	—	—	0·23	0·39	—	0·39	—
*20-2 or 3	—	—	3·68	—	—	—	—	—	—
20-3	0·56	0·57	1·07	1·6	1·1	1·6	0·89	2·13	1·8
20-4	9·0	7·0	10·4	20·2	18·2	26·1	14·3	21·0	21·0
22-5	—	1·25	Trace	1·6	0·51	2·2	—	0·56	—
22-6	1·02	2·8	2·6	6·9	5·4	7·2	2·2	4·2	5·6

* Tentative identification only.

expressed in terms of the dry weight of either the cell sap or the liver pulp. This relationship expresses the richness of the cell sap, as compared with the liver as a whole, in particular lipid fractions, and if the lipids were evenly distributed on a weight basis these would be unity. It is evident that, in these terms, the cell sap is enriched only in the cholesterol esters, and is particularly poor in phospholipid (fractions 5, 6 and 7).

Fatty acid composition of the cell sap

The data in Table 4 show that the total fatty acids of the cell sap are only slightly less saturated than those of the liver pulp (Getz *et al.* 1961). However, there are large differences within the individual lipid classes. Thus the sterol ester fraction of the cell sap contains much more stearate and arachidonate, and less oleate and docosahexanoate, than does the corresponding fraction of the pulp. Both the tri- and lower-glyceride fractions have more stearate and arachidonate and less unsaturated C_{18} esters than are in the liver pulp.

The phospholipids of the cell sap have compositions similar to those of the liver pulp. Within the cell sap itself the phospholipids contain less palmitate and C_{18} unsaturated acids than do the neutral lipids. The highest arachidonate content is found in the inositide-containing fraction. Docosahexaenoic acid is approximately evenly distributed between the silicic acid fractions 4, 5 and 6. By contrast, in the whole-liver pulp this acid was found at highest concentration in the amino phosphatide (chloroform–methanol, 4:1) fraction.

The lipid fraction of the cell sap eluted from the silicic acid column by the chloroform–methanol (7:1) mixture might be expected to contain cardiolipin by analogy with the corresponding fraction obtained from the whole-liver pulp. However, the low linoleate and the high arachidonate and stearate of the cell-sap fraction suggest that cardiolipin is only a minor component.

The material which was insoluble in chloroform and which was removed from the lipid extract before the silicic acid chromatography had a fatty acid composition similar to that of the 'sphingolipid' fraction.

Composition of the 'floating fatty supernatant'

In a previous study of the fatty acid composition of the 'floating fatty supernatant' separated from a rat-liver homogenate, it was shown to resemble closely that of the adipose tissue (Getz & Bartley, 1961). The present data (Table 6) show that this fraction, also like the adipose tissue, contains over 90% of its fatty acid esters in the form of neutral glycerides. Relative to the cell lipids as a whole, the neutral lipids are markedly concentrated and the phospholipids are much diluted in this fraction. The sterol esters are slightly more concentrated than in the whole cell but not as greatly as in the cell sap (cf. Table 3). The data in Table 5 show that the triglyceride fraction eluted from the silicic acid column had a high content of free fatty acids. These fatty acids resembled quite closely those fatty acids present in the triglycerides but differed in particular in the greater amount of palmitate present (Table 7). They do not therefore arise entirely as a result of triglyceride hydrolysis during the fractionation procedure.

Phospholipids were present in very low concentrations in the 'floating fatty supernatant'. The analysis of the various phospholipid fractions eluted from the silicic acid columns (Table 5) suggests that the elution pattern was anomalous. For this reason, the fatty acid composition of the various lipid fractions containing phosphorus which were obtained (Table 7) cannot easily be related to that of the phospholipid fractions of the cell sap or of the whole liver.

Metabolic lability of the 'floating fatty supernatant'

Experiments were carried out to determine whether the fatty acids of the 'floating fatty supernatant' were rapidly exchanged with the fatty acids of the diet. A 'floating fatty supernatant' fraction was prepared from the livers of

Table 5. *Analysis of lipid fractions from 'fatty supernatant' of rat liver*

Results are expressed as μmoles/g. dry wt. of 'fatty supernatant'.

Fraction no.	Esters (μmoles)	Free acid (μmoles)
1	3·9	18·2
2	321·0	109
3	52·2	3·8
4	6·6	—
5	9·9	—
6	1·0	1·9
7	6·7	8·2
8	1·9	—

Table 6. *Distribution of lipids within the 'fatty supernatant' compared with the mean distribution throughout the cell*

For explanation of *A* and *B* see Table 3.

Fraction no.	Percentage distribution	*A*	*B*
1	0·97	1·12	0·26
2	92·50	23·8	5·5
3	1·63	1·9	0·45
4	2·44	0·77	0·18
5	0·26	0·17	0·038
6	1·67	0·18	0·042
7	0·46	3·76	0·79
Total	—	1·18	5·11

Table 7. *Fatty acid composition of the 'floating fatty supernatant' of rat liver*

Values are given as percentages of the total methyl esters (see Table 4). br, branched chain; h.br, highly branched; uns, unsaturated (see Table 4).

Fatty acid	Total acids	Sterol esters	Tri-glycerides	Free acids	Lower neutral glycerides	Fraction eluted with chloroform–methanol (7:1)	Amino phosphatides	Inositides	Chloroform-insoluble fraction	Lecithin	Sphingo-lipid
9-0	—	—	—	—	0·06	—	—	—	—	—	—
10-0	—	—	—	—	0·02	—	0·17	—	—	—	—
11-0	—	—	0·09	—	0·05	—	0·62	—	—	—	1·04
12-0	0·06	0·15	0·18	0·09	0·09	1·41	1·74	—	—	0·21	1·59
*13-h.br	—	—	—	—	0·03	—	—	—	—	—	—
13-br	—	—	—	—	—	—	—	—	0·58	—	—
13-0	—	—	—	—	—	—	—	—	—	—	1·18
*14-h.br	—	0·35	0·12	0·08	0·11	0·94	0·96	—	7·0	0·30	—
14-0	1·1	0·77	0·88	1·48	0·72	1·33	1·51	1·49	4·7	0·80	1·51
14-1	—	0·21	0·07	0·07	0·28	—	0·23	—	1·75	0·16	—
*15-br	—	0·24	—	—	0·08	—	1·17	—	—	—	—
15-br	0·56	0·24	—	0·09	0·13	1·00	0·92	—	0·64	0·13	1·8
15-0	—	0·44	—	0·72	0·28	1·17	1·22	0·72	1·9	0·53	1·32
*16-h.br	—	0·51	—	—	—	—	1·08	—	1·36	—	—
*16-h.br	—	—	0·66	0·09	0·37	10·7	6·6	—	—	0·35	6·9
16-0	26·0	16·3	22·3	31·3	21·7	30·0	27·6	20·2	23·8	27·7	22·0
16-1	2·9	5·3	2·8	3·1	2·4	2·3	2·15	2·6	5·6	1·59	2·5
*17-h.br	—	—	—	—	0·18	—	0·63	—	—	—	—
17-0	0·39	0·24	0·18	0·25	0·26	—	0·83	1·14	—	0·81	—
17-br	0·31	1·16	0·41	0·36	0·43	1·31	1·12	1·6	1·41	0·54	0·75
*17-uns	—	—	—	0·32	—	—	—	—	—	0·28	—
*18-h.br	—	0·27	0·21	0·24	0·27	—	0·74	—	—	—	—
18-0	2·0	1·8	1·5	1·45	1·39	6·1	13·3	20·2	20·8	19·6	21·3
18-1	28·0	35·3	31·9	28·4	31·3	20·9	14·6	18·4	16·1	12·9	10·4
18-2	27·0	23·2	29·6	27·3	32·6	15·6	12·2	16·6	7·7	16·7	7·1
18-3	0·56	0·55	0·22	1·6	—	—	—	—	—	—	—
*18-1	—	—	—	—	—	—	—	0·87	—	—	—
*19-?	—	—	—	—	—	—	—	1·19	—	—	—
*19-h.br	—	0·21	—	—	—	—	—	0·45	—	—	—
*19-br	0·66	—	0·06	—	0·20	—	—	—	—	0·47	—
19-0	—	—	0·18	—	—	—	—	—	—	—	—
20-0	0·55	—	—	0·23	—	—	—	—	—	—	—
20-1	0·76	—	0·74	0·43	0·59	—	—	—	—	—	—
20-2	0·45	—	0·27	0·36	0·58	—	—	—	—	—	1·6
20-3	0·55	0·76	0·46	0·46	0·81	—	—	1·68	—	1·25	—
20-4	2·0	10·7	1·6	1·5	2·4	2·1	7·8	12·8	6·7	13·6	7·3
20-5	0·51	0·62	0·59	—	—	—	—	—	—	—	—
21-uns	0·83	—	—	—	—	—	—	—	—	—	11·8
22-5	1·4	—	1·11	0·83	1·21	2·2	—	—	—	Trace	—
22-6	3·1	0·76	2·9	1·01	1·72	2·9	3·0	—	—	2·06	—

* Tentative identification only.

Table 8. *Fatty acid distribution in 'fatty supernatant' of rats with and without corn oil in the diet*

Values are given as percentages of the total methyl esters (see Table 4).

Fatty acid	Corn oil	Control	After feeding with corn oil
11-0	—	0·04	—
12-0	0·6	0·17	0·08
14-0	0·7	1·16	1·14
14-1	—	—	0·10
15-0	0·2	0·38	0·48
16-br	—	0·23	—
16-0	15·4	25·06	24·6
16-1	0·23	4·69	4·2
*17-uns	—	0·38	0·38
17-0	—	—	0·36
*18-h.br	—	—	0·25
18-0	4·0	4·6	3·2
18-1	21·2	34·2	33·4
18-2	35·5	22·2	29·6
18-3	—	0·97	—
20-1	1·9	—	0·59
20-3	—	—	0·40
20-4	—	1·48	1·33
22-1	—	—	—
22-5	5·0	—	—
22-6	—	—	0·87
Others	15·3	—	—

* Tentative identification only.

eight rats that had been fed with 1 ml. of corn oil 4 hr. before they were killed. The fatty acid composition of this fraction was compared with that of a 'floating fatty supernatant' prepared from the livers of seven rats on their normal diet. The results are shown in Table 8. Little change occurred in the fatty acid pattern of the fraction after feeding with corn oil.

DISCUSSION

The present work is believed to provide the first detailed investigation of the lipid composition of the cell-sap fraction in a particular organ, although isolated studies on particular tissue-lipid components have been described previously. Ada (1949) reported that 9 % of the cell phospholipids were present in the cell sap. Our values are very much lower than this. The data of Kretchmer & Barnum (1951) on the mouse-liver soluble fraction show a low proportion of phospholipid (16·7 %), but the soluble fraction contained 12 % of the cell fat whereas our value was 4·8 %. It must be borne in mind that these authors ground their tissue in alkaline salt solution instead of sucrose before isolation of the cytoplasmic fractions. They also report a high percentage of choline-containing phospholipids. Marinetti, Erbland & Stotz (1958) found that neutral lipids made up 54·4 % of the fat of a soluble fraction corresponding to the cell sap prepared from pig heart whereas our value for rat-liver-cell sap is 65·3 %. Lecithin formed 52·4 % of the total phosphatides of the pig-heart soluble fraction but only 40 % of the rat-liver-cell sap was lecithin.

Sphingomyelin is a large constituent of the pig-heart soluble fraction and, as judged in this study by the amount of the lipid fraction eluted with chloroform–methanol (1:4), it is also a main constituent of the rat-liver-cell sap.

Rice, Schotz, Alfin-Slater & Deuel (1953) and Schotz, Rice & Alfin-Slater (1953) have investigated the distribution of cholesterol and cholesterol esters in the rat-liver cell. They used young female rats of a different strain. They reported their 'F' layer, corresponding to the 'floating fatty supernatant' of this study, to contain 62 % of the recovered sterol esters of the whole liver. Our figures are very much lower than this.

The high glyceride content of the cell-sap fraction may be due at least in part to contamination with triglyceride from the 'floating fatty supernatant'. This is particularly rich in triglyceride. Of some interest is the apparent lack of rapid equilibration between dietary fatty acids and those of the 'floating fatty supernatant'. From other studies it is known that the quantity of dietary fatty acids which enters the liver in an acute experiment, such as that described, would be sufficient to replace the fatty acids of this fraction several times over (see Robinson & French, 1960). The minimal changes in the composition of the fatty acids of the 'floating fatty supernatant', which were observed after feeding with a fat of characteristic fatty acid composition, such as corn oil, suggest that such exchange does not occur to any marked extent. Recent studies of Stein & Shapiro (1959), who found that [1-^{14}C]palmitate injected intravenously was incorporated into the triglycerides of the 'floating fatty supernatant' much less rapidly and extensively than into those of the mitochondria and microsomes, support this view.

SUMMARY

1. The cell sap of the rat liver has been separated, the constituent lipids fractionated on silicic acid and their fatty acid composition determined.

2. A similar study has been carried out on the 'floating fatty supernatant' of the rat liver.

3. Of the total liver fatty acid esters 4·6 % were in the cell sap and 5·1 % of the esters in the 'fatty supernatant'. Both these fractions were largely neutral lipid. About one-third of the cell sterol esters were in the cell sap and about the same proportion of the total lipid in this fraction was sterol ester. Over 90 % of the 'floating fatty supernatant' was triglyceride, representing about one-quarter of the total cell triglycerides.

4. Experiments in which corn oil was fed to rats showed that the 'fatty supernatant' did not rapidly equilibrate with the dietary fat.

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Effect of Carbohydrates on the Intestinal Synthesis of Thiamine in Rats

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The intestinal synthesis of thiamine depends on dietary constituents, which affect both the type and number of intestinal micro-organisms (Kuno, 1953; Johansson, 1956; Balakrishnan, Baliga & Rajagopalan, 1957; Mameesh & Johnson, 1958; Nath & Meghal, 1960).

Banerji (1941) observed that the presence of much protein in the basal diet prevented the bradycardia and loss of weight usually associated with thiamine deficiency. Banerji & Yudkin (1942) reported that rats grow and thrive well in the absence of thiamine, provided that they do not have to metabolize dietary carbohydrate. According to Banerji & Yudkin (1942) and Yudkin (1951) the signs of thiamine deficiency are due to the toxic products of disturbed carbohydrate metabolism. As little as 10% of glucose in the diets led to polyneuritis and death (Yudkin, 1951), and sorbitol has been shown to be thiamine-sparing (Morgan & Yudkin, 1957; Jones & Baumann, 1958).

When potato starch is added to the thiamine-free basal diet, rats can grow well and this phenomenon was described by Fridericia, Freudanthal, Gudjohnson, Johanssen & Schaubye (1927) as 'refection'. According to them the end products of fermentation lowered the pH of the intestinal contents and this appeared to promote the growth of micro-organisms which synthesize vitamins of the B group. Nagase & Fugita (1956) reported that cellulose helps in the intestinal synthesis of thiamine in man.

Therefore, we have studied the effect of different carbohydrates on the faecal coliform count and daily excretion of thiamine in the urine and faeces of rats. Hydrolysed 'glucose cycloacetoacetate', which has been shown to contain glucose (1:2-dienol) and has a strong reducing action even in acid medium (Nath & Bhattathiry, 1956), not only helps in prevention of experimental diabetes (Nath & Behki, 1957*a*, *b*; Nath & Bhattathiry, 1959) and experimental hyperlipaemia induced by high concentrations of saturated fats or cholesterol in the diet (Nath & Saikia, 1959*a*, *b*), but also stimulates the intestinal biosynthesis of thiamine in rats kept on a thiamine-free diet (Nath & Meghal, 1960). Therefore we also studied the effect of hydrolysed 'glucose cycloacetoacetate' on the thiamine deficiency induced by adding either glucose or sucrose to a thiamine-free basal diet.

EXPERIMENTAL

Forty-eight male weanling albino rats (30–40 g. each) were divided into eight groups and placed individually in wire-bottomed cages. Coprophagy was not prevented. A carbohydrate-free basal diet, deficient in thiamine (Morgan & Yudkin, 1957), was used and the additional carbohydrates were given by replacing the same weight of casein. The composition of the thiamine-free diets given to the different groups of animals is shown in Table 1. Each rat received the respective diet *ad lib.* and daily dietary intake was noted.

The animals were given the appropriate diets for 6 weeks. After the first 4 days in each week, urine and faeces were collected for the remaining 3 days of the week and the thiamine content was determined by the method of Mawson & Thompson (1948). Weekly weights of animals were also recorded.

During the second, fourth and sixth weeks the faecal bacterial count was measured in three rats from each group. The faeces of each rat were collected aseptically on the last 3 days of the week in weighed sterile test tubes and a uniform suspension of about 0·1 g. of faeces with 10 ml. of 0·9% NaCl soln. was made under aseptic conditions. Further dilutions were made with the saline and the coliform colonies in the faeces were counted by the plate method in Mackonkey's agar medium (Balakrishnan & Rajagopolan, 1952).

At the end of the sixth week the animals were weighed and killed. The thiamine contents of liver, kidney, brain and thigh muscle were determined by the method of Kratzing & Slater (1950).

The thiamine content of honey estimated by the thiochrome method of Harris & Wang (1941) was 6·4 μg./100 g. 'Glucose cycloacetoacetate' was prepared by condensing glucose with ethyl acetoacetate as described by West (1927) and hydrolysed according to the method of Nath & Sahu (1953). The aqueous extract of hydrolysed material, after repeated washing with ether, was diluted with water so that it contained the equivalent of 80 mg. of 'glucose cycloacetoacetate'/ml.

RESULTS

Daily dietary intake. The daily dietary intake of rats belonging to groups 1 (controls), 4, 6 and 8 gradually increased, and the dietary intake of those belonging to groups 2, 3 and 5 decreased (Table 2). The daily thiamine intake from honey (group 2) was 0·29–0·32 μg./rat.

Urinary and faecal excretion of thiamine. From the values in Table 3, it can be seen that the weekly urinary and faecal excretion of thiamine of groups 1 (controls), 4, 6 and 8 was higher than that of all other groups, thereby giving an indication of increased rate of thiamine synthesis throughout the entire experimental period; the rats in group 2 showed a slight increase up to the fourth week in the excretion of thiamine in urine and faeces. After the fourth week it decreased. As the rats in groups 3 and 5 died after the fourth week owing to thiamine deficiency, their faecal and urinary excretion of thiamine could be estimated only during the first 4 weeks and it was found to be decreased. The rats in groups 3 and 5 showed loss of appetite and polyneuritic symptoms. The pectin-

Table 1. *Composition of thiamine-free diets*

The diet for each group of rats consisted of vitamin-free casein 70%, salt mixture no. 185 (McCollum & Davis, 1915) 5%, groundnut oil 15% and the addition (10%) indicated below. Each rat also received daily: riboflavin 80 μg., nicotinic acid 500 μg., pyridoxine hydrochloride 40 μg., calcium panthothenate 250 μg., biotin 0·2 μg., *p*-aminobenzoic acid 250 μg., folic acid 10 μg., choline chloride 5 mg., inositol 2 mg., α-tocopherol acetate 30 μg., vitamin K 50 μg., vitamin B_{12} 0·2 μg. Vitamins A and D were given twice weekly in the form of Adexolin [Glaxo Laboratories (India) Ltd. Bombay] 84 mg.

Group	Addition
1 (Control)	Casein
2	Honey
3	Glucose
4*	Glucose
5	Sucrose
6*	Sucrose
7	Cellulose
8	Gum acacia (pectin)

* These groups also received hydrolysed 'glucose cycloacetoacetate' (80 mg./rat/day).

Table 2. *Average daily dietary intake (g.) of rats in each group*

For group diet see Table 1.

Group	Week no. 1	2	3	4	5	6
1	3·5	3·8	4·2	4·5	5·0	5·0
2	3·10	4·6	3·7	3·2	4·0	4·2
3	4·0	4·2	3·8	3·9	—	—
4	3·9	4·8	5·1	5·2	5·1	4·8
5	4·8	3·8	3·9	4·2	—	—
6	3·6	3·8	3·9	4·2	4·6	4·5
7	3·6	4·6	5·7	5·2	5·0	5·0
8	4·5	5·0	5·5	5·7	5·7	5·8

Table 3. *Effect of carbohydrates on weekly urinary and faecal excretion of thiamine by rats*

Values are expressed in μg./rat/24 hr. mean ± S.D. For group diet see Table 1.

Group	Urinary excretion in week					
	1	2	3	4	5	6
1	0·78±0·02	0·85±0·05	0·98±0·06	1·63±0·08	2·33±0·05	2·35±0·06
2	0·81±0·04	0·89±0·06	0·93±0·03	1·21±0·01	0·88±0·02	0·88±0·06
3	0·55±0·06	0·43±0·03	0·35±0·06	0·28±0·07	—	—
4	0·63±0·03	0·85±0·05	1·66±0·03	1·88±0·05	2·08±0·06	2·13±0·04
5	0·61±0·05	0·63±0·02	0·31±0·07	0·20±0·03	—	—
6	0·85±0·07	0·99±0·01	1·35±0·02	1·83±0·06	2·16±0·03	2·18±0·04
7	0·85±0·03	0·96±0·03	1·21±0·04	0·85±0·05	0·55±0·03	0·55±0·03
8	0·83±0·04	0·93±0·05	1·61±0·05	1·81±0·03	1·95±0·02	2·20±0·03
	Faecal excretion in week					
	1	2	3	4	5	6
1	1·21±0·09	1·35±0·03	1·36±0·05	1·81±0·06	2·39±0·03	2·43±0·01
2	1·01±0·03	1·21±0·02	1·26±0·03	1·00±0·02	0·89±0·03	1·00±0·06
3	0·33±0·06	0·51±0·03	0·37±0·05	0·35±0·06	—	—
4	1·18±0·01	1·35±0·09	1·89±0·09	2·09±0·04	2·10±0·28	2·12±0·18
5	0·88±0·06	0·75±0·06	0·55±0·08	0·21±0·03	—	—
6	1·21±0·06	1·35±0·18	1·89±0·09	2·12±0·12	2·28±0·11	2·30±0·18
7	0·86±0·02	1·05±0·07	1·12±0·03	1·00±0·02	0·89±0·03	1·00±0·06
8	1·23±0·06	1·35±0·07	1·65±0·09	1·75±0·06	2·11±0·09	2·06±0·02

Table 4. *Faecal coliform count of groups of three rats*

Average values are expressed in 1000/g. of wet faeces. For group diet see Table 1.

Group	Week no.		
	2	4	6
1	60·0	80·0	103·0
2	62·0	25·0	8·0
3	16·0	5·3	—
4	57·3	95·8	99·0
5	30·0	10·8	—
6	49·0	88·0	102·0
7	50·0	32·0	12·0
8	82·0	95·0	108·0

fed group (group 8), however, showed a constant increase in the urinary and faecal excretion of thiamine; the cellulose-fed group (group 7) showed a slight increase in the urinary and faecal excretion of thiamine during the first 4 weeks, after which it decreased.

Faecal coliform bacteria. The faecal coliform organisms were increased in groups 4, 6 and 8 and their number was similar to that of the controls (Table 4).

Examination of the relative coliform counts in the faeces of rats of the eight groups brings out the following points: (1) The coliform organisms were suppressed by the ingestion of glucose and sucrose throughout the experiment and the animals belonging to these groups died of thiamine deficiency in the fifth week. (2) Hydrolysed 'glucose cycloacetoacetate' had the capacity to overcome the harmful effect of glucose and sucrose on the intestinal flora and this beneficial effect is seen in groups 4 and 6 as the coliform count in the faeces of these groups increased gradually. (3) The coliform count in groups 2 and 7 was nearly the same as in the control group during the second week, after which it showed a gradual decrease. (4) The pectin-fed group (group 8) showed a constant increase in the faecal coliform count during the experiment, thereby indicating the increase in the intestinal synthesis of thiamine.

Weekly weight of animals. The animals of groups 1 (controls), 4, 6 and 8 showed a gradual increase in weight during the experiment; but glucose- and sucrose-fed rats showed a loss of weight and ultimately died of thiamine deficiency. Honey- and cellulose-fed rats showed no significant change in body weights (Table 5).

Tissue storage of thiamine. The content of thiamine in different tissues gives an indication of the state of health of the rats and the efficiency of the dietary factors in contributing to the body reserve. A comparison of the thiamine contents of liver, brain, kidney and muscle of eight groups (Table 6) shows that rats belonging to the groups 1 (controls), 4, 6 and 8 had greater storage of thiamine than did other groups.

DISCUSSION

The results of this experiment support the view that the animals receiving only 10% of glucose or sucrose in their thiamine-free diet suffer from

Table 5. *Effect of carbohydrates on the weekly weights of rats*

Mean values in g. ± S.E.M. For group diet see Table 1.

Group	Initial wt.	Week 1	2	3	4	5	6
1	36±2·1	42±2·8	48±1·6	59±2·7	62±1·9	64±2·0	69±5·6
2	39±3·1	42±2·2	50±3·1	48±2·6	46±3·7	43±5·1	44±3·8
3	35±3·2	36±2·8	39±4·2	39±2·3	38±3·1	—	—
4	38±4·5	44±2·8	56±5·2	59±3·7	62±2·6	71±4·7	72±5·2
5	30±2·2	31±3·8	34±2·2	32±1·6	30±2·5	—	—
6	37±1·8	40±2·1	52±2·7	56±3·1	66±2·5	70±3·9	74±4·2
7	32±2·2	39±1·8	40±3·8	42±1·8	38±1·9	36±1·2	36±1·5
8	32±1·5	39±1·2	48±2·8	64±3·2	66±2·3	68±3·2	68±1·7

Table 6. *Effect of carbohydrates on tissue thiamine content after feeding for six weeks*

Mean values in μg./100 g. ± S.E.M. For group diet see Table 1.

Group	Liver	Brain	Kidney	Muscle
1	64·1±0·096*	86·4±1·20*	42·2±1·88*	24·5±1·88*
2	29·8±0·64	34·3±1·24	16·6±0·52	14·2±1·06
3	—	—	—	—
4	68·7±0·97*	81·7±0·82*	49·7±0·68*	23·4±0·89*
5	—	—	—	—
6	62·3±0·81*	69·9±2·43*	31·8±1·41*	21·6±1·53*
7	16·7±1·43	22·6±0·85	17·4±0·89	10·6±0·65
8	60·4±0·78*	73·8±3·61*	52·4±2·8*	25·2±1·06*

* Difference from group 2 values are significant at $P < 0·01$.

thiamine deficiency (Yudkin, 1951), as their thiamine excretion in urine and faeces is very much reduced as compared with other groups. The coliform organisms in the faeces of these groups were very much reduced, showing that these two substances might have a suppressing effect on the intestinal flora. In such experiments, coprophagy plays an important role. In our experiment, coprophagy was allowed. Morgan & Yudkin (1959) showed that sorbitol was effective in preventing thiamine deficiency in rats only when coprophagy was allowed. They further showed that, when coprophagy was prevented in sorbitol-fed rats, the rats suffered from thiamine deficiency. These workers also observed that the thiamine-deficient rats fed on glucose excreted in the faeces an amount of thiamine that was too small to prolong the life of such rats even for a short time. The hydrolysed 'glucose cycloacetoacetate', which enhances the biosynthesis of thiamine (Nath & Meghal, 1960), overcame the harmful effect of glucose or sucrose when it was fed along with a thiamine-free basal diet. In the honey-fed group there was also a decrease in urinary and faecal excretion of thiamine but the animals of this groups did not suffer from thiamine deficiency. Honey contains about 80% (w/w) of reducing sugars (Loesecke, 1949) and therefore the rats of this group would have been expected to show the signs of thiamine deficiency; this may have been prevented by the presence of thiamine or thiamine-sparing substances in the honey.

When potato starch is fed, rats can grow well in the absence of thiamine (Fredericia *et al.* 1927). Barnes, Kwong, Delany & Fiala (1960) did not confirm this; they showed that the inclusion of dextrin as carbohydrate source in diets deficient in thiamine permitted enhanced growth but the effect of dextrin was completely abolished when coprophagy was prevented. In our experiment, the effect of cellulose can be compared with that of dextrin because the cellulose-fed rats showed no signs of thiamine deficiency. Nagase & Fugita (1956) have shown that cellulose helps in the intestinal synthesis of thiamine in man. The present work shows that cellulose did not have the harmful effect of glucose or sucrose.

The pectin-fed group showed a constant rise in the urinary and faecal excretion of thiamine, thereby indicating the enhancing effect of pectin in the biosynthesis of thiamine. The pectin is formed of a branched chain of D-galactose, L-arabinose, L-rhamnose and D-glucuronic acid. The pectin undergoes hydrolysis in the process of digestion and the substances mentioned above are liberated; this may have a favourable effect on the intestinal flora, thus increasing the faecal thiamine that is ultimately available to rats after coprophagy.

SUMMARY

1. Eight groups of rats were given thiamine-free diet containing 70% of protein, with additional protein (10%) or carbohydrate (10%), e.g. honey, glucose, sucrose, cellulose or pectin. The amount of hydrolysed 'glucose cycloacetoacetate' given in combination with glucose or sucrose was 80 mg./rat daily.

2. Rats receiving either glucose or sucrose only suffered from thiamine deficiency and died during the experiment.

3. Hydrolysed 'glucose cycloacetoacetate' overcame the harmful effect of glucose or sucrose and the rats of these two groups showed an increase in urinary and faecal thiamine excretion, maintaining a constant increase in weight during the experiment. The tissue storage of thiamine in these rats was also increased.

4. Rats fed on honey and cellulose showed no significant change in urinary and faecal excretion of thiamine.

5. Urinary and faecal excretion of thiamine was increased in pectin-fed rats and their tissue thiamine content was also greater than that of honey- or cellulose-fed groups.

6. The faecal coliform count in control groups fed with hydrolysed 'glucose cycloacetoacetate' or with pectin was higher than in honey- and cellulose-fed groups.

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